Tampere University

ANA ANDJELKOVIĆ

Expression of Alternative Oxidase Influences Cell Migration

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ACADEMIC DISSERTATION
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ABSTRACT

Cell migration is important in animal development, tissue repair, the functioning of the immune system and for tissue homeostasis in general. Impairments in cell migration are associated with various developmental abnormalities and pathologies. In *Drosophila* development, cell migration is instrumental during metamorphosis, in the process of thoracic closure. The most studied mammalian model of cell migration at the cellular level is the scratch or wound-healing assay, in which a linear scratch is made in a confluent monolayer of cells, which then migrate to close the gap.

The work presented in this thesis aims to understand how signaling associated with the mitochondrial respiratory chain affects cell migration in different models. As a tool to investigate this relationship, I used model organisms transgenically expressing the alternative oxidase, AOX, from a primitive marine animal, the tunicate *Ciona intestinalis*. AOX is an accessory component of the mitochondrial respiratory chain, which is found in microbes, plants, and some metazoan phyla, but not in vertebrates or insects. AOX directly oxidizes ubiquinol by molecular oxygen in a non-proton motive reaction, by-passing respiratory chain complexes III and IV. Utilizing AOX from *Ciona intestinalis*, I perturbed the mitochondrial respiratory chain and investigated the effect on developmental signaling affecting cell migration in the *Drosophila* and mammalian cell models. To create a control for these studies and test whether the ability of AOX to alleviate tested phenotypes depends on its enzymatic activity, I engineered a mutated variant of AOX in such a way as to abolish this activity.

I observed that co-expression of AOX from *Ciona intestinalis* was able to alleviate cleft thorax and other dysmorphic phenotypes in *Drosophila*, brought about by activated GeneSwitch transcription factor, which I hypothesize to interfere in some way with nuclear receptor signaling during development. Using the mutant AOX control, I was able to show that AOX enzymatic activity is instrumental in rescuing developmental lethality or locomotor dysfunction, resulting from cytochrome oxidase deficiency. I proceeded to use mutant AOX to show that the same is true for the rescue of the dysmorphic phenotypes induced by GeneSwitch.

AOX expression also alleviated the cleft thorax phenotype induced by genetic manipulations of the JNK signaling pathway, which regulates the formation of the dorsal thoracic epithelium and governs the migratory behavior of the cell sheets everting from the wing imaginal discs during metamorphosis.

Midline closure defects similar to cleft thorax in the fly are also seen in mammals, for example, in spina bifida, cleft lip and palate and cleft sternum. Considering the well conserved biology between fly and human and the highly conserved JNK signaling pathway, it was possible to use a mammalian cell-culture model to test the generality of the findings from *Drosophila*, as well as explore the molecular mechanisms underlying the influence of AOX on cell migration. I thus used the mammalian wound-healing model to confirm that AOX expression promotes migration in immortalized (but not primary) mouse embryonic fibroblasts, and rescues pharmacologically induced migration deficiencies through a mechanism involving JNK signaling. Reporter assays showed that AP-1 and its transcriptional activity are not a direct target of AOX. However the data suggest a possible direct involvement of JNK, acting through other targets.

Despite the lack of knowledge on how AOX is regulated in animals, the use of various inhibitors showed that the effect of AOX on cell migration is most likely due to a specific effect on metabolism, possibly due to its thermogenic activity. A full elucidation of the processes that link mitochondrial perturbations with cell migration should be of considerable medical importance and might even enable the design of new and more effective treatments, e.g., for metastatic tumors, tissue injuries, and congenital midline closure defects. A better understanding of the role mitochondria play in mediating cellular signaling is still needed, and will be instrumental to fully understand many fundamental biological processes, causes of disease and enable the design of precision treatments.

Results from this thesis provide us with a new paradigm linking mitochondrial function with developmental cell signaling. They also highlight what can be learned by combining tools and findings from different model organisms. In this case, the tunicate *Ciona intestinalis* provided a tool to better understand complex developmental processes in *Drosophila melanogaster*, which was then followed up in mammalian cells with potential relevance to human diseases.

TIIVISTELMÄ

Solujen liikkumisella eli migraatiolla on tärkeä rooli yksilönkehityksessä, kudosvaurioiden korjaamisessa, immuunijärjestelmässä sekä yleisesti kudosten toiminnassa. Solumigraation ongelmat liittyvät moninaisiin kehityshäiriöihin ja sairauksiin. *Drosophila melanogaster* -banaanikärpäsellä puutteellinen solujen migraatio yksilönkehityksen aikana näkyy muun muassa halkiona kärpäsen keskiruumiissa. Solumigraation tutkituimmassa solutason nisäkäsmallissa yksikerroksiseen solupeitteeseen tehdään lineaarinen viilto, joka umpeutuu solumigraation seurauksena.

Väitöskirjatyöni tavoitteena oli tutkia, miten mitokondrioiden soluhengitykseen liittyvä signalointi vaikuttaa solumigraatioon erilaisissa tutkimusmalleissa. Tutkimuksessani hyödynsin mallieläintä, joka oli geneettisesti muokattu tuottamaan alkukantaisesta *Ciona intestinalis* -vaippaeläimestä eristettyä vaihtoehtoista oksidaasia (alternative oxidase, AOX). AOX-entsyymiä ei esiinny selkärankaisilla eikä hyönteisillä, mutta muun muassa mikrobeilla, kasveilla ja joillakin monisoluisilla eläimillä se toimii osana mitokondrion hengitysketjua hapettamalla ubikinolin suoraan käyttäen molekulaarista happea. AOX ohittaa entsyymikompleksit III ja IV ilman protonien kuljetusta. Tutkimuksessani muokkasin mitokondrioiden luontaisen hengitysketjun toimintaa hyödyntäen AOX:ia sekä banaanikärpäs- että nisäkässolumallissa, ja tutkin tämän vaikutuksia yksilönkehityksen aikaiseen signalointiin ja solujen migraatioon. Kokeiden kontrolliksi ja testatakseni johtuuko fenotyypin oireiden lieventyminen AOX:n entsymaattisesta aktiivisuudesta, muokkasin AOX:stä mutaation avulla entsymaattisesti toimimattoman version.

Havaitsin, että *Ciona intestinalis*ista saadun AOX:n lisääminen kärpäsmalliin lievensi aktivoidun GeneSwitch transkriptiofaktorin aiheuttamaa keskiruumishalkiota ja muita kehityshäiriöitä. Hypoteesinani on, että aktiivinen GeneSwitch häiritsee jollakin mekanismilla tumareseptorien viestintää kehityksen aikana. Käyttämällä mutatoitua AOX-kontrollia osoitin, että sytokromioksidaasin vajeesta johtuvia letaaleja kehityshäiriöitä ja liikuntakyvyn ongelmia lieventävä vaikutus perustuu AOX:n entsymaattiseen aktiivisuuteen. Jatkoin käyttämällä mutatoitua AOX:ää osoittaakseni saman pätevän myös GeneSwitchin tuottamiin kehityshäiriöihin. AOX:n ilmentäminen lievensi lisäksi keskiruumishalkiota, joka

aiheutui JNK-signalointireitin geneettisestä manipuloinnista. JNK-signalointireitti säätelee keskiruumiin dorsaalisen epiteelin muodostumista ja ohjaa siipiaihioiden solukerrosten migraatiota kärpäsen muodonmuutoksen aikana.

Nisäkkäillä on banaaninkärpäsen keskiruumishalkion kaltaisia keskilinjan kehityshäiriöitä, kuten selkäranka-, huuli-, suulaki- sekä rintalastahalkioita. Monet biologiset toiminnot ja varsinkin JNK-solusignalointireitti ovat säilyneet samankaltaisina ihmisellä ja banaanikärpäsellä, mikä mahdollistaa nisäkässolumallien käytön kärpäsillä saatujen tulosten yleispätevyyden kokeilemiseen sekä AOX:n vaikutusten taustalla olevien molekulaaristen mekanismien tutkimiseen. Näin ollen pystyin hyödyntämään nisäkässoluviljelmään tehtyä lineaarista viiltoa menetelmänä osoittaakseni, että AOX:n ilmentäminen edesauttaa solumigraatiota immortalisoiduissa (mutta ei primaarisissa) hiiren alkion sidekudossoluissa ja korjaa JNK-reitin manipulaation aiheuttamia häiriöitä solujen migraatiossa. Reportterimenetelmät paljastivat, että transkriptiotekijä AP-1 ja sen aktiivisuus eivät ole AOX:n suora kohde, vaan AOX:lla on mahdollisesti suora yhteys JNK-reitin toimintaan jotakin muuta kautta.

Huolimatta siitä, että tietous AOX:n säätelymekanismeista eläimillä on puutteellista, kokeet useilla eri inhibiittoreilla viittasivat siihen, että AOX:n vaikutus solujen migraatioon todennäköisimmin selittyy spesifillä vaikutuksella aineenvaihduntaan; mahdollisesti entsyymin termogeenisellä toiminnalla. Kattavan kokonaiskuvan saaminen prosesseista, joiden kautta mitokondriaaliset häiriöt liittyvät solumigraatioon, tarjoaisi merkittävää lääketieteellistä tietoutta ja saattaisi jopa auttaa muun muassa syövän etäpesäkkeiden, kudosvaurioiden ja synnynnäisten keskilinjan kehityshäiriöiden hoitomuotojen kehittämisessä. Tarvitaan kuitenkin parempaa käsitystä mitokondrioiden osuudesta solusignaloinnissa, ja tämä on olennaista monien biologisten prosessien, sairauksien syiden ymmärtämisen ja kohdennettujen hoitojen suunnittelun kannalta.

Väitöskirjassani esitetyt tutkimustulokset tarjoavat uuden mallin, joka liittää mitokondrioiden toiminnan yksilönkehityksen aikaiseen solusignalointiin. Tuloksissani korostuu myös se, mitä voidaan oppia yhdistelemällä erilaisten mallien tarjoamaa tutkimustietoa ja -menetelmiä. Tässä tapauksessa *Ciona intestinalis* -vaippaeläimen käyttö auttoi ymmärtämään paremmin *Drosophila melanogaster*in monimutkaista yksilönkehitystä. Näiden tulosten pohjalta pystyin jatkamaan tutkimusta mitokondrioiden roolista solujen migraatiossa nisäkässolumallissa, millä on potentiaalisesti merkitystä ihmisen sairauksien tutkimisessa.

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ABBREVIATIONS

20E	20-hydroxyecdysone
4-HNE	Hydroxy-2-nonenals
ADH	Alcohol dehydrogenases
ADP	Adenosine diphosphate
AKRs	Aldo-keto reductases
ALDH	Aldehyde dehydrogenase
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AOX	Alternative oxidase
AP	Apurinic/apyrimidinic sites
AP-1	Activator protein 1
AS601245	Inhibitor V; inh V
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
basket	*bsk*
BSA	Bovine serum albumin
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
*Ci*AOX	*Ciona intestinalis* alternative oxidase
complex I	NADH:ubiquinone oxidoreductase
complex II	Succinate:ubiquione oxidoreductase
complex III	Ubiquinol:cytochrome *c* oxidoreductase
complex IV	Cytochrome *c* oxidase
complex V	ATP synthase
COX	Cytochrome *c* oxidase
cyt *c*	Cytochrome *c*
DC	Dorsal closure
dNF-Y	Nuclear transcription factor Y
dp	Dorsal prothoracic disc

Dpp	Decapentaplegic
ECM	Extracellular matrix
EcR	Ecdysone receptor
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase 1 and 2
ETF	Electron-transferring flavoprotein
ETF	Electron-transferring flavoprotein
ETS-1	E26 avian erythroblastosis virus transcription factor
GDNF	Glial cell line-derived growth factor
GPCR	G protein coupled receptor
GPx	Glutathione peroxidases
GS	GeneSwitch System
GSH	Gluthatione
H_2O_2	Hyrogen peroxide
HClO	Hypochlorous acid
hemipterus	*hep*
HPV16	Human papillomavirus 16
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
hTERT	Human telomerase reverse transcriptase
Httex1p Q93	Human huntingtin with 93 polyglutamine repeats residue
iMEFS	Immortalized mouse embryonic fibroblasts
IMM	Inner mitochondrial membrane
JNK	c-Jun N-terminal kinase
Jra	Jun-related antigen
kay	*kayak*
KD	Ketogenic diet
kDa	Kilodalton, measure of molecular weight or mass
KGDHC	α-ketoglutarate dehydrogenase enzyme complex
LE	Leading-edge
LPO	Lipid peroxidation product
MAP3K	Mitogen-activated protein kinase kinase kinase
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase-activated protein kinase

MAPKK	Mitogen-activated protein kinase kinase
MEFs	Mouse embryonic fibroblasts
misshapen	*msn*
MitoQ	Mitoquinone mesylate
MMPs	Matrix metalloproteinases
MnSOD	Manganese-dependent superoxide dismutase
mtDNA	Mitochondrial DNA
MTND4	Fourth subunit of NADH dehydrogenase enzyme encoding gene
mutAOX	Mutated variant of AOX
NAC	N-acetyl cysteine
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
Ndi	NADH dehydrogenase (alternative)
NF-κB	Nuclear factor kappa B
NRF2	NF-E2-related factor 2
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide ion radical
O_3	Ozone
$OH^{\bullet}$	Hydroxyl radical
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase complex
PDM	Peridroplet mitochondria
PGCs	Primordial germ cells
Pi	Inorganic phosphate
PM	Plasma membrane
PMA	Phorbol-12-myristate-13-acetate
pnr	*pannier*
puc	*puckered*
PUFAs	Polyunsaturated fatty acids

pvr	PDGF and VEGF receptor related
Q	Ubiquinone
QH_2	Reduced ubiquinone, ubiquinol
RC	Respiratory chain
Ref-1	Redox factor 1
RNAi	Ribonucleic acid interference
$RO^{\bullet}$	Alkoxyl radicals
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RTK/ERK	Tyrosine kinase/extracellular regulated kinase
RU486	Antiprogestin mifepristone
RXR	Retinoid X receptor
Scrib	Scribble complex
SOD	Superoxide dismutase
SOD1	Cytoplasmic superoxide dismutase
SOD2	Mitochondrial superoxide dismutase
SOM	Sister-of-Mammalian Grainyhead
TAO	*Trypanosoma* alternative oxidase
*Tb*AOX	*Trypanosoma brucei* alternative oxidase
TCA	Ticarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TGFβ-1	TGFβ-1 transforming growth factor beta
tTG	Tissue transglutaminase
UAS	Upstream activation sequence
UPR	Mt mitochondrial unfolded protein response
Usp	Ultraspiracle
VEGF	Vascular endothelial growth factor
w/v	Weight/Volume
Wg	Wingless
wt	Wild-type

ORIGINAL PUBLICATIONS

Publication I **Andjelković**, A., Oliveira, M. T., Cannino, G., Yalgin, C., Dhandapani, P. K., Dufour, E., Rustin, P., Szibor, M., & Jacobs, H. T. (2015). Diiron centre mutations in *Ciona intestinalis* alternative oxidase abolish enzymatic activity and prevent rescue of cytochrome oxidase deficiency in flies. *Scientific Reports*. https://doi.org/10.1038/srep18295

Publication II **Andjelković, A**., Kemppainen, K. K., & Jacobs, H. T. (2016). Ligand-Bound GeneSwitch Causes Developmental Aberrations in *Drosophila* that Are Alleviated by the Alternative Oxidase . *G3: Genes, Genomes, Genetics* https://doi.org/10.1534/g3.116.030882

Publication III **Andjelković, A**., Mordas, A., Bruinsma, L., Ketola, A., Cannino, G., Giordano, L., Dhandapani, P. K., Szibor, M., Dufour, E., & Jacobs, H. T. (2018). Expression of the Alternative Oxidase Influences Jun N-Terminal Kinase Signaling and Cell Migration. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.00110-18

1 INTRODUCTION

Regulation of epithelial sheet migration is a critical component of animal development. For example, dysregulation of epithelial gap closure results in birth defects including spina bifida and cleft palate. Incorrectly regulated cell migration can also lead to impaired wound healing and to metastasis in cancer. The signaling pathways which regulate cell migration are incompletely understood. Previous work by other groups has suggested a role for mitochondrial metabolism in this process. The goal of this research was to identify mitochondria-related targets for manipulating cell migration and epithelial morphogenesis, which may enable future treatments of birth defects and other disorders.

In this work, I applied a genetic approach in the model fruit fly *Drosophila melanogaster* to dissect the signaling pathways regulating epithelial morphogenesis and explore the effects of altering mitochondrial metabolism. In both humans and fruit flies, the migration of cell sheets is known to be regulated by the JNK signaling pathway. Thorax closure depends upon morphogenetic movements during *Drosophila* development where migrating epithelial sheets everting from the wing imaginal discs join together to form the dorsal thoracic epithelium. Defects in cell signaling impair this process and produce a cleft thorax phenotype in flies, roughly analogous to spina bifida or cleft palate in humans.

Alternative respiratory pathways are present in many eukaryotic organisms including some arthropods; but are not present in insects or vertebrates. Their roles include (1) to promote thermogenesis, (2) to prevent accumulation of reducing equivalents, and (3) to act as antioxidants and provide resistance to metabolic poisons. One of these alternative pathway enzymes is the alternative oxidase (AOX), which bypasses complexes III and IV of the mitochondrial respiratory chain.

Here, I expressed the AOX gene from the tunicate *Ciona intestinalis* in flies that had been genetically modified to exhibit cleft thorax. I selected two such models. In the first of these I made use of the steroid-dependent GeneSwitch transcriptional activator, which I observed to induce cleft thorax and other dysmorphic adult phenotypes in the presence of the inducing drug RU486. The project was initiated by the chance observation that AOX expression was able to reverse these effects. In

the second model, I generated cleft thorax by specifically downregulating components of the JNK pathway.

To enable these studies, I began by creating an important control for these and all studies involving the expression of an exogenously derived AOX. I engineered mutant AOX lacking enzymatic activity to test if the phenotypic effects of AOX expression are due to enzymatic activity or some other property conferred by AOX.

The identification of effectors that rescue cleft thorax in *Drosophila* may eventually translate to humans. Therefore, to build upon the *Drosophila* findings, I also explored the effect of AOX expression on JNK-dependent transcription and wound healing in cultured mammalian cells.

The finding that AOX can influence cell sheet movements in different organisms implies that a general relationship exists between mitochondrial respiratory function and cell migration that might one day find applications in medicine.

2 REVIEW OF THE LITERATURE

2.1 Mitochondria

The mitochondrion is an organelle present in most cells of most eukaryotes. The only exceptions are some species of fungi (Margulis, 1981), and some anaerobic microbial eukaryotes, such as *Pelomyxa palustris* (Brandt & Pappas, 1959) or the oxymonad *Monocercomonoides* (Karnkowska *et al.*, 2016). In mammals mitochondria are not found in differentiated erythrocytes. Mitochondria were first described as elementary organisms living inside the cell by Altman (1890), who called them bioblasts. The term 'mitochondrion' was first used by Benda (1897), who detailed their unique shape. According to the endosymbiotic theory, eukaryotes evolved from free living oxygen-metabolizing α-protobacteria, which were engulfed by a pre-eukaryotic scavenger cell some 1.6 billion years ago (Zimorski *et al.*, 2014). However, the pre-eukaryotic cell did not digest the α-protobacteria, but rather provided it shelter and nourishment, establishing symbiosis (Margulis, 1981; Lane & Martin, 2010).

2.1.1 Mitochondria: structure and function

The number of mitochondria per cell varies between different cell types and organisms. Kinetoplastida, and some apicomplexans, such as *Toxoplasma*, have a single mitochondrion, while the oocytes of most animals, as well as the giant amoeba *Chaos chaos* contain thousands (Bereiter-Hahn & Voth, 1994). In a typical human cell there are 100-1000 mitochondria, depending on cell-type and its metabolic status. Each cell contains about 1000 nucleoids, which are supramolecular assemblies of mitochondrial DNA (mtDNA) compacted by mitochondrial transcription factor A (TFAM) (Brown *et al.*, 2011; Kukat *et al.*, 2015) and associated with other proteins. Mammalian mtDNA encodes only 13 polypeptides, each of which contribute to the oxidative phosphorylation (OXPHOS) complexes.

Each mitochondrion has four interconnected compartments: two membranes, the outer (OMM) and inner (IMM) mitochondrial membranes; the intermembrane

space between them and the mitochondrial matrix (MM). The OMM and IMM differ in their composition and permeability (Daum &Vance, 1997; de Kroon *et al*., 1997; Mejia & Hatch, 2016). The IMM contains the mitochondrial respiratory chain (RC) which is composed of five enzyme complexes.

The OMM includes the following proteins: enzymes involved in the metabolism of amino acids and fatty acids, receptor complexes for protein import (Lill & Neupert, 1996), pore forming proteins (Benz, 1994), proteins controlling mitochondrial morphology (Sogo & Yaffe, 1994), signal transduction components (deKroon *et al*., 1997), the machinery for the import and export of lipids part of which remains unknown (deKroon *et al*., 1997), and monoamine oxidases A and B (Schnaitman *et al*., 1967; Edmondson *et al*., 2009). In eukaryotes, cardiolipin is the only lipid that is fully synthesized in the mitochondrion. The IMM has several invaginations called cristae. Cristae are dynamic and their number and structure may have functional consequences (Bereiter-Hahn & Jendrach, 2010).

Many mitochondrial enzymes which are involved in the above-mentioned pathways are localized in the matrix but some of them are tightly connected with the IMM, e.g., succinate dehydrogenase, a component of the tricarboxylic acid cycle (TCA cycle), dihydroorotate dehydrogenase, which functions in the pyrimidine nucleotide biosynthesis pathway, and choline dehydrogenase). TCA cycle enzymes are bound to each other and to the IMM forming a supramolecular complex called the metabolon (D'Souza & Srere, 1983; Velot *et al*., 1997). It was proposed that the organization of these enzymes into such a metabolon allows efficient intermediate transport between active sites. The first structural evidence of substrate channeling in the TCA cycle metabolon was shown by Wu & Minteer (2015). Only part of the energy released by the oxidation of respiratory substrates is used to produce adenosine triphosphate (ATP) and to transport metabolites, while the rest is released as heat. Mitochondria serve as heat generators in brown fat tissue which is found in all mammals (Smith, 1964; Cannon & Nedergaard, 2004). Mitochondria themselves function at temperatures at least 6 to 10 °C higher than the rest of the cell (Chrétien *et al*., 2018).

Mitochondria are not stationary organelles. They are able to change structurally as well as move to sites of high adenosine triphosphate (ATP) demand to meet the energy requirements of the cell. Locomotion of the mitochondria and changes in their shape reflect the locations of energy consumption in the cell (reviewed by Bereiter-Hahn & Voth, 1994).

Mitochondria have a set of well-defined functions. These differ according to cell type and are performed by mitochondria being able to change their number,

localization, shape and molecular components. Mitochondria generate energy in the form of ATP, using a so-called proton-motive force formed by three of the RC complexes (described in the next section).

Mitochondria are responsible for regulating cell death and survival (Tournier *et al.*, 2000; Schell *et al.*, 2014; Morita *et al.*, 2017) and also play vital roles in calcium homeostasis (Rasola *et al.*, 2010; Lim *et al.*, 2008) and steroid synthesis (Miller *et al.*, 2013). There are several important metabolic processes (or parts thereof) localized in the mitochondria including: the TCA, urea cycle, beta-oxidation of fatty acids (though not in all eukaryotes), haem synthesis, glycine cleavage and folic acid metabolism. The generation of iron-sulfur clusters is catalyzed by mitochondrial scaffold proteins and enzymes (Rouault & Tong, 2005). Six classes of steroid hormones (calciferols (Vitamin D), glucocorticoids, mineralocorticoids, estrogens, progestins and androgens), are made from cholesterol in mammalian mitochondria by mitochondrial P450 enzymes (Miller, 2013). Mitochondria achieve all of this by communicating with many different cell organelles including the endoplasmic reticulum and sarcoplasmatic reticulum in tight connection with the cytoskeleton, which is crucial for mitochondrial localization and translocation to the site of action. Mechanobiologists have furthermore proposed that the extracellular matrix mechanics also influence mitochondrial function (Bartolák-Suki *et al.*, 2017; Lyra-Leite *et al.*, 2017). In fat-oxidizing tissues mitochondria associate with lipid droplets as peridroplet mitochondria (PDM). The function of PDMs is still largely unknown. Oocyte mitochondria in many species are organized together with Golgi bodies, endoplasmic reticulum and other organelles into a Balbiani body (Raven, 1961).

Rapid transport of the mitochondria has been shown to occur along neuronal axon microtubules (Boldogh & Pon, 2007). Animal mitochondria rely primarily on microtubules for their transport, although there is evidence that actin filaments are also involved in their motility (Ligon & Steward, 2000). Mitochondrial movement in budding yeast is dependent on the actin cytoskeleton, not microtubules (Boldogh & Pon, 2007). Plants and other fungi also rely on actin filaments for mitochondrial transport. Mitochondrial localization inside the cell is not random and is important in physiology and disease (Campello & Scorrano, 2010). For example, in cell migration, anterior localization of mitochondria is necessary for the cells to migrate faster with greater persistence (Desai *et al.*, 2013). Interestingly, perturbing mitochondrial localization within cells by mutating mitochondrial fusion and fission proteins impacts the distribution of mitochondria, decreases the number of cells with anterior-localized mitochondria and slows cell migration (Desai *et al.*, 2013).

In most tissues mitochondria appear to be a dynamic network which can change their shape and location in the cell (Bereiter-Hahn & Voth, 1994). Mitochondrial movement is coordinated with changes in their shape, as shape has to be compatible with their movement (de Vos *et al.*, 2005). Their shape depends on their function, cell-cycle stage, fusion and fragmentation. Mitochondria can change shape from elipsoid to interconnected tubular organelles. Continuously ongoing fusion and fission processes are instrumental in determining mitochondrial shape (Campello & Scorrano, 2010). Mislocalization of the mitochondria is important in a number of neurodegenerative diseases (Ferreirinha, 2004; Baloh, 2007) and it can affect lifespan. For example, decreasing mitochondrial fission has been shown to increase lifespan and fitness of *Podospora anserina* and *Saccharomyces cerevisiae* (Scheckhuber *et al.*, 2007). The morphology of mitochondria is important for their function. For example, malformed, 'donut-shaped' mitochondria, a hallmark of the mitochondrial stress (Liu & Hajnóczky, 2011), are associated with abnormally small synaptic contacts, affecting the working memory in rhesus monkeys (Hara *et al.*, 2014).

Mitochondria are crucial to the functions of many differentiated cells in animals and have tissue specific functions. For example mitochondria are involved in regulation of synaptic transmission, brain function and cognition in aging (Sharpley *et al.*, 2012; Hara *et al.*, 2014). Sperm cell metabolism is also highly dependent on mitochondria. Although paternal mitochondria are degraded inside the fertilized egg, sperm mitochondria are critical for fertilization and sperm function (Koppers *et al.*, 2008). Sperm cells are vulnerable to oxidative stress (Mueller & Robaire, 1996; Aitken *et al.*, 2012; Selvaratnam & Robair, 2016) and a correct mitochondrial redox homeostasis balance is crucial for normal sperm motility (Amaral *et al.*, 2013). Moreover, sperm mitochondria in humans are protected in a keratinous structure, the mitochondrial capsule, formed by disulfide bonds between cysteine- and proline-rich selenoproteins, including the sperm-specific phospholipid hydroperoxidase glutathione peroxidase (Ursini *et al.*, 1999; Amaral *et al.*, 2013). Mitochondria are also major components in the glucose-sensing mechanism controlling insulin secretion by pancreatic β-cells (Lowell & Shulman, 2005; Maassen *et al.*, 2004; Maechler & Wollheim, 2001). Another example of a tissue specific function is the production of ketone bodies, as important energetic substrates, occurring exclusively in mammalian liver mitochondria. Finally, mitochondria serve as critical regulators of autophagy via their role in redox and metabolic homeostasis (Engel & Evans, 2006; Scherz-Shouval *et al.*, 2007; Chen *et al.*, 2007; Azad *et al.*, 2009).

2.1.2 The mitochondrial respiratory chain

Biochemical energy from nutrients is converted into ATP by glycolysis and the reactions of cellular respiration, combined with ATP synthase, later two making up the system of OXPHOS. OXPHOS is a key functional unit in the mitochondria. Respiration is performed by RC, which comprises NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquione oxidoreductase, often described more simply as succinate dehydrogenase (complex II), ubiquinol:cytochrome *c* oxidoreductase (bc1 complex; complex III), cytochrome *c* (Cyt *c*), and cytochrome *c* oxidase (CcO; complex IV). In most tissues, the majority of electrons transferred by the RC are derived from NADH and enter the chain via complex I. Electrons derived from $FADH_2$ in complex II feed directly into the ubiquinone (Q) pool. Q is lipid soluble and freely moves through the hydrophobic core of the membrane. Once reduced, (QH_2), ubiquinone delivers its electrons to the next complex in the electron transport chain. Electrons are transferred through the RC to oxygen, while the mitochondrial membrane potential is generated by pumping of protons across the IMM. In the final step, ATP synthase (complex V) utilizes the proton gradient to energize the production of ATP from ADP and Pi. Importantly, the respiratory chain also sustains the TCA cycle by re-oxidizing NADH and $FADH_2$.

2.1.3 Mitochondrial dysfunction and disease

The term 'mitochondrial disorders' is used for a collection of clinical syndromes characterized by faulty OXPHOS. The first mitochondrial disorder was described by Luft *et al.*, (1962). He reported a case of a 35 year-old woman with general weakness, excess perspiration, high skin temperature, and inability to gain weight. Mitochondria of the patient exhibited defects in mitochondrial enzyme organization and had densely packed cristae with tubular inner structures (Luft *et al.*, 1962). Mitochondrial disorders can result from deficiency or dysfunction of any OXPHOS component. Over 200 different genes are involved in assembling the OXPHOS complexes (Smeitink *et al.*, 2001), and loss or functional inhibition of any of them can inhibit OXPHOS. Respiratory chain complexes can also be inhibited by various toxins that target the complexes directly, e.g., I (rotenone, Lindahl & Öberg, 1961), III (antimycin, Slater, 1973), IV (cyanide, Van Heyningen, 1935), or V (oligomycin, Bruni & Luciani, 1962).

The incidence and prevalence of mitochondrial disorders is difficult to estimate because of their clinical and genetic variability and the limitations of current

diagnostic techniques (Skadal *et al.*, 2003). Mitochondrial disorders manifest as a heterogeneous clinical presentation, from single tissue disorders such as specific neuropathies and myopathies, to multi-system disorders. The onset of the disease can occur at any life stage from neonatal to late adult (Zeviani & Di Donato, 2004). It is estimated that mitochondrial disorders occur with a frequency of 1/5,000 to 1/10,000 births (Smeitink *et al.*, 1998). Mutation analysis is complicated by the complexity of the mitochondrial respiratory chain, which is composed of 13 subunits encoded by mtDNA and over 70 subunits encoded by nuclear DNA. There are over 300 mtDNA mutations associated with mitochondrial disorders (Govindaraj *et al.*, 2011). Mutations of mtDNA can be large-scale rearrangements, partial deletions or duplications, but are usually sporadic, inherited point mutations (Zeviani & Di Donato, 2004). A large number of other nuclear genes are required for mitochondrial protein import and assembly, as well as regulation of mtDNA replication and expression (Shoubridge, 2001). In all, some 1,500 nuclear genes code for mitochondrial proteins (Chinnery & Hudson, 2013), any of which is potentially involved in mitochondrial disease. Thus, mitochondrial disease can be caused by mutations in either the nuclear or mitochondrial genome and can be inherited as autosomal dominant/recessive, X-linked or maternally. Use of next generation sequencing has made the diagnosis and understanding of mitochondrial diseases better, which will lead to treatments (Nightingale, 2013). However, in approximately 50% of adult patients with biochemical and morphological evidence of a mitochondrial disorder, the affected gene remains unidentified (Zeviani & Di Donato, 2004). Even the same mitochondrial disease, such as Leigh syndrome, can be genetically heterogeneous. In some cases it is caused by mtDNA mutations in ATP synthase subunit 6 (Makino *et al.*, 2000) or NADH dehydrogenase subunit 4 (Hadzsiev *et al.*, 2010). In others it is due to autosomal recessive nuclear gene mutations in SURF1, LRPPRC (a mitochondrial mRNA-binding protein), complex I subunits NDUFS7 or NDUFS8 or complex II subunit SDHA (Zeviani & Di Donato, 2004).

Tissues which require energy the most, such as the visual and auditory systems, the CNS and PNS, the heart, muscle, endocrine pancreas, kidney and liver are most sensitive to OXPHOS failure (Zeviani & Di Donato, 2004), and are most commonly affected in mitochondrial disease.

Mitochondria and mtDNA are maternally inherited in all mammals (Hutchinson *et al.*, 1974; Sato & Sato, 2013) and does not undergo germline recombination (Hällberg & Larsson, 2014). However, paternal mitochondria carried by sperm do enter the egg during fertilization (Schwartz & Vissing, 2002). During mammalian

zygote formation, sperm mitochondria are removed by either ubiquitination post fertilization (Sutovsky *et al.*, 1996; Sato & Sato, 2013) or potentially during transport through the male reproductive tract (Sutovsky, 2003). However, two reports of paternal transmission of mitochondria, suggest that paternal mtDNA could be passed to the offspring in specific cases (Schwartz & Vissing, 2002; Luo *et al.*, 2018). Mitochondria and their genomes are believed to be randomly distributed to daughter cells during cell division (Jenuth *et al.*, 1996). However, stem-like cells have been observed to distribute mitochondria unequally, based on the age of the mitochondria (Katajisto *et al.*, 2015). This was only observed within the stem-like cells in the culture and it is unknown if this phenomenon occurs *in vivo* (Sun *et al.*, 2016). However, there is evidence of a corresponding asymmetric inheritance of both mitochondria in asymmetric division in yeast (McFaline-Figueroa *et al.*, 2011).

A repair system not as efficient as that in the nucleus, the proximity of the mtDNA to reactive oxygen species (ROS) production sites and the fact that mtDNA packing by TFAM is not as protective as the nuclear DNA by the histones associations, have been proposed to cause a higher rate of mutation in mtDNA compared to nuclear DNA (LeDoux *et al.*, 2007; Alexeyev *et al.*, 2013). This causes heteroplasmy, where, in a single cell or mitochondrion, wild-type and mutated mtDNA can coexist. Heteroplasmic mutations will not always manifest clinically. Only when mutated gene copies accumulate over a certain threshold, will the effects of the mutation no longer be masked by the co-existing wild-type mtDNA, and disease symptoms will become apparent (Thorbum & Dahl, 2001). However, the critical threshold differs depending on the exact mutation site, tissue, increasing over time in post-mitotic tissues and decreasing in mitotic tissues such as blood. mtDNA deletions give rise to a lower heteroplasmic threshold (~50%) for the appearance of disease symptoms (Rossignol *et al.*, 2003). For many common point mutations, a level of 80-90% mutant mtDNA needs to be reached before symptoms manifest (Chinnery *et al.*, 1997).

Mitochondrial disorders currently have no cure. Mostly they are progressive, leaving the patient with severe disability and shortened lifespan. However, disease progression can also halt and symptoms may stay stable for decades. The complexity of mitochondrial diseases and the fact that they are still incurable necessitates different approaches to treatment. A hallmark of mitochondrial diseases is decreased ATP synthesis. Therefore, some therapeutic interventions aim at increasing the levels of ATP (Viscomi *et al.*, 2015). Antioxidant treatments are also employed with the goal of protecting cells from increased oxidative damage caused by mitochondrial dysfunction (Ni *et al.*, 2016; He *et al.*, 2014). Endurance exercise and small molecule

compounds such as vitamins and cofactors have also been used to improve cellular energy metabolism or enhance it indirectly by inducing mitochondrial biogenesis (Reznick & Shulman, 2006; Jager *et al.*, 2007; Holloway, 2009). Exercise improves endurance and muscle function and can also increase the percentage of healthy, non-mutated mtDNA as well as trigger mitochondrial biogenesis (Viscomi *et al.*, 2016). Nutritional therapy, with focus on implementing a ketogenic diet (KD) might be beneficial for mitochondrial function and alleviate mitochondrial disorders (Kerr, 2010; Pfeffer *et al.*, 2013; Peralta *et al.*, 2015). KD is based on high fat, moderate protein and low carbohydrates. Ketone bodies produced from the oxidation of fat are the main source of energy (de Lima *et al.*, 2014). On KD, cells are forced to bypass glycolysis and use oxidative phosphorylation. Preclinical research shows that KD induces mitochondrial biogenesis slowing down disease progression in the Deletor mouse, which has a mutant form of the mitochondrial helicase TWINKLE, which causes progressive external ophthalmoplegia (Ahola-Erkkila *et al.*, 2010). Ketogenic medium has been shown to kill cybrid cell lines, which carry 100% deleted mtDNA (Santra *et al.*, 2004).

Furthermore, the idea that increasing the amount and/or function of mitochondria could be beneficial to treat mitochondrial disease, has been tested in several ways. Treating fibroblasts from patients with different mitochondrial diseases with a pan-PPAR agonist, bezafibrate (Bastin *et al.*, 2008), corrected respiratory complex deficiency in patient cells (Bastin *et al.*, 2008). Replacing damaged mitochondria with autologous respiration-competent mitochondria has been reported to recover myocardial dysfunction (Emmani & McCully, 2018). In some patients with sparing myopathies, the pathogenic mtDNA is surprisingly absent in terminally fated myogenic precursor cells named satellite cells (Smith *et al.*, 2004). Therefore, myotoxins have been used to destroy mature muscle myofibres harboring the mtDNA mutation, which leads to repopulation of the muscle by satellite cells (Clark *et al.*, 1997). For diseases caused by heteroplasmic mtDNA mutations, one approach seeks to increase the proportion of wild-type mtDNA, using gene therapy to eliminate mutated mtDNA and propagate the wild-type mtDNA (Taylor *et al.*, 2000). Examples of such an approach include the use of a mitochondrially targeted restriction endonuclease designed to preferentially eliminate mutated mtDNA (Bacman *et al.*, 2012). Another version uses sequence-specific nucleic acids which selectively bind and induce mutant mtDNA degradation (Mukherjee *et al.*, 2008). However, despite many ingenious strategies to transfect DNA or RNA into mitochondria (Seibel *et al.*, 1995), none has been shown to work routinely *in vivo*.

Genetic counseling in a family with a history of mitochondrial diseases is important, given the severity of the mitochondrial diseases. However, the inheritance of mutant mtDNA cannot be reliably predicted because of the germ cell bottleneck effect, wherein a limited number of mtDNA molecules are transferred into each oocyte during primary oocyte generation. That means that mothers with pathogenic mtDNA mutations will have offspring with variable levels of mutated mtDNA which cannot be predicted in advance (Chinnery, 2000). However, this can be evaluated using *in vitro* fertilization followed by preimplantation genetic diagnosis (Herbert & Turnbull, 2018). Women affected with high levels of heteroplasmy or homoplasmy, where nearly all mtDNA is mutated, could in the future undertake a form of germline therapy, mitochondrial replacement therapy, which would greatly decrease the risk of transmitting disease. Mitochondrial replacement therapy requires that an enucleated egg from an unaffected donor to be transplanted with the nuclear genome from the mother affected with the mitochondrial disease (Herbert & Turnbull, 2018). Environmental factors can also trigger or aggravate mitochondrial diseases (Wallace, 2010; Cheng *et al.*, 2010), which adds additional complexity to treatment.

Among these are pharmaceuticals (Wallace, 2010; Stumpf & Copeland, 2014), as well as exposure to environmental chemicals, such as rotenone, cyanide and other RC inhibitors which can interact with the genetic risk factors and trigger or aggravate the disease.

Mitochondrial dysfunction has been implicated in several psychiatric diseases including autism spectrum disorders (Prabakaran *et al.* 2004; Rossignol & Frye, 2012), and neurological disorders such as Rett syndrome (Dotti *et al.*, 1992). Five percent of children with autism spectrum disorder meet mitochondrial disease criteria (Rossignol & Frye, 2012). Such associations are extremely important as patients may benefit from treatments focused on addressing mitochondrial functionality. Mitochondrial dysfunction has also been linked with infertility (reviewed in Wallace, 1999). mtDNA mutations are found in a wide spectrum of cancers (Polak *et al.*, 1998; Ishikawa *et al.*, 2001; Copeland *et al.*, 2002; Tan *et al.*, 2002), but it remains unclear if the mtDNA mutations influence carcinogenesis and if mtDNA plays a crucial role in the development of cancer. More work is needed to understand the significance of specific mitochondrial mutations in cancer and disease progression. (Ishikawa *et al.*, 2001; Chatterjee *et al.*, 2006). Acquired mtDNA mutations and mitochondrial dysfunction are also proposed to be involved in aging and age-related diseases such as diabetes (Mootha *et al.*, 2003). Mitochondrial dysfunction is also observed in chronic periodontitis (Govindaraj *et al.*, 2001; Pallavi *et al.*, 2016).

2.1.4 mtROS and their role in cell signaling

Oxygen and any other compound potentially capable of accepting electrons is described as a pro-oxidant. In biological systems the most important pro-oxidants are ROS. ROS are byproducts of aerobic life (Davies, 2000) and can be split into two groups of compounds, radicals and nonradicals. Radicals include the nitric oxide (NO), superoxide ion ($O^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). The group of nonradical compounds considered as ROS include hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3), and O_2 itself (Kohen & Nyska, 2002). Low amounts of ROS are necessary to maintain different cell signaling systems (Schieber & Chandel, 2014; Mittler, 2017). In the 1980s, Sies (1985, 1986, 1991) proposed the oxidative stress concept. He described it as an imbalance between oxidants and antioxidants in favor of the former. The high reactivity of ROS makes them perfect signaling molecules (reviewed by D'Autréaux & Toledano, 2007), influencing many biological processes, such as proliferation (Szatrowski & Nathan, 1991; Preston *et al.*, 2001), apoptosis, immunity, defense against microorganisms and autophagy (reviewed by Scherz-Shouval & Elazar, 2007).

ROS produced by mitochondria are termed mtROS. They act as signaling molecules in both normal physiology and in disease etiology (Cadenas & Davies, 2000; Finkel & Holbrook, 2000; Schieber & Chandel 2014; Mittler, 2017).

There are many oxidative stress-responsive transcription factors and genes and some of these have been implicated in influencing the aging processes. The effect of ROS on expression and activity of transcription factors is complex and occurs at multiple levels. For example, although ROS generally cause an increase in AP-1 and NF-$\varkappa$B levels (Pinkus *et al.*, 1996), oxidative stress can at the same time decrease the transcriptional activity of these molecules through the direct oxidation of critical cysteine residues contained within the DNA-binding domain (Schenk *et al.*, 1994; Meyer *et al.*, 2015). The concentration of ambient oxygen influences embryonic development (Allen, 1991) as well as ROS generation (Turrens *et al.*, 1992). Superoxide dismutase (SOD) activity increases during human fetal development in liver, blood and placenta, and during differentiation of monocytes (Allen, 1998). Macrophages and neutrophils create ROS which serve as bactericidal, anti-viral, and anti-tumor agents (Lander, 1997). Autophagy also depends on ROS (reviewed in Scherz-Shouval & Elazar, 2007; Moore, 2008; Azad *et al.*, 2009) and, in turn, serves to decrease oxidative damage (Wu *et al.*, 2009; Yang *et al.*, 2014).

The RC is the major source and paradoxically the major target of ROS. Even though the RC is considered to be very efficient in passing electrons, several iron-sulfur clusters within the respiratory chain are exposed and vulnerable to toxic, side reactions with oxygen and can produce superoxide ($O_2^{\bullet-}$) (reviewed by Cadenas & Davies, 2000). As a by-product of normal RC activity, the RC is able to participate in one-electron reduction of oxygen leading to the formation of superoxide anion-radical. As mentioned, the most susceptible sites for ROS damage are thiol groups and iron-sulfur clusters. The latter are specifically damaged by superoxide anion-radicals, which are mainly produced at complex I, which contains ROS-sensitive thiols and many iron-sulfur clusters (Turrens *et al.*, 1980) as well as complex III, which also contains iron-sulfur clusters (Finkel & Holbrook, 2000). Similar damage can also affect complex II (Quinlan *et al.*, 2012; Moreno-Sánchez *et al.*, 2013) and other sites in the mitochondrion. The α-ketoglutarate dehydrogenase enzyme complex (KGDHC) in brain mitochondria produces ROS in the mitochondrial matrix when the NADPH/$NADP^+$ ratio there is elevated (Starkov *et al.*, 2004). Complexes IV and V do not contain flavins or iron-sulfur clusters and thus are not sites of ROS production.

ROS need to be well regulated in order to prevent oxidative damage in the cell. As signaling molecules, ROS can oxidize factors in a variety of pathways that lead to growth and survival, by altering enzyme activity, cellular localization, and protein-protein interactions (reviewed by Schieber & Chandel, 2014). This involves different molecular targets, including protein kinases (Nogueira *et al.*, and phosphatases (Hecht & Zick, 1992; Hamanaka & Chandel, 2010), transcription factors (Kohlgrüber *et al.*, 2017), e.g., nuclear factor kappa B (NF-ϰB) (Flohé *et al.*, 1997; Pateras *et al.*, 2014), NF-E2-related factor 2 (NRF2) (Mimura & Itoh, 2015), activator protein 1 (AP-1) (Abate *et al.*,1990; Amstad *et al.*, 1992), E26 avian erythroblastosis virus transcription factor-1 (ETS-1) (Shiu *et al.*, 2018), Sister-of-Mammalian Grainyhead (SOM) involved in neural tube closure, wound healing, and epithelial cell migration, cell-cycle regulators and membrane lipids (Gustavsson *et al.*, 2007; Caddy *et al.*, 2010) and many others.

Redox changes work through oxidation or reduction of protein sulfhydryls which induces conformational changes. In turn this alters the properties of proteins, such as DNA binding activity (Abate *et al.*, 1990, Hecht & Zick, 1992), interactions with regulatory subunits, or the formation of protein complexes, which may be necessary for signal transduction or transcription to proceed.

The mitochondrial RC controls a number of physiological and pathological cellular responses in part by producing mtROS. Much of this superoxide is rapidly

converted to H_2O_2 by mitochondrial superoxide dismutase (SOD2) as well as by cytoplasmic superoxide dismutase (SOD1). H_2O_2 is able to oxidize cysteine residues in proteins to cysteine sulfenic acid or to form disulfide bonds within or between proteins (Schieber & Chandel, 2014). This can upregulate cell signals generated, for example, by growth factors and hormones such as insulin (Cheng *et al.*, 2010; Loh *et al.*, 2009). Oxidative deactivation of catalytic cysteines in the active sites of protein tyrosine phosphatases like the tumor suppressor PTEN influences cell migration, growth, and survival (Lee *et al.*, 2002; Hopkins *et al.*, 2014).

2.1.4.1 ROS-induced molecular damage

Accumulation of ROS leads to oxidative stress where cellular constituents, including proteins, DNA and lipids are oxidized and suffer damage. The protonated form of the superoxide anion and the hydroxyl radical commonly initiate the process of autocatalytic lipid peroxidation. ROS react with the acyl moiety of lipid molecules. Polyunsaturated fatty acids are more susceptible to ROS attack, since the hydroperoxyl radical reacts more readily with acyl chains compared to a carbon-carbon double bond. 4-hydroxy-2-nonenals (4-HNE) is considered a biomarker for oxidative stress and mediates a number of signaling pathways (Zhong & Yin, 2015). The net result of lipid peroxidation is conversion of unsaturated lipids into polar lipid hydroperoxides, which causes increased membrane fluidity, efflux of cytosolic solutes and loss of membrane-protein activities (Avery, 2011). Brain mitochondria have a higher concentration of lipids with polyunsaturated acyls, which are therefore more sensitive to free radical oxidation than any other lipids (Bolanos *et al.*, 1997). Oxidation of the mitochondrial phospholipid cardiolipin leads to formation of 4-HNE and other oxidation products (Yin & Zhu, 2012; Zhong *et al.*, 2014). Oxidation of cardiolipin in turn has diverse consequences: it regulates apoptosis (Kagan *et al.*, 2005), leads to mitochondrial dysfunction, mitophagy (Chu *et al.*, 2013) and human diseases (Paradies *et al.*, 2009).

Ribo- and deoxyribonucleic acids are also subjected to oxidative damage. DNA damage can be grouped into: strand breaks (single and double), sister chromatid exchange, DNA-DNA and DNA-protein cross-links, and base modifcations. All four bases can be altered which may lead to apurinic/apyrimidinic (AP) sites. ROS-mediated DNA sugar and phosphate damage creates strand breaks (reviewed in Davies, 2000). DNA damage itself can result in elevated ROS generation (Hamanaka & Chandel, 2010; Kang *et al.*, 2012). Direct oxidation of side chains of cysteine, tyrosine, histidine, arginine, and lysine residues, which are among the most

susceptible to ROS damage, results in the addition of reactive carbonyl functional groups on proteins, of which aldehydes are the most reactive (Suzuki *et al.*, 2010). Oxidation of enzymes often leads to their inactivation. However, not all enzymes are equally susceptible to oxidation. For instance, glucose 6-phosphate dehydrogenase is considered to be among the most susceptible (Lushchak & Gospodaryov, 2005).

2.1.4.2 ROS defense mechanisms in the cell

ROS production is a by-product of oxidative metabolism. Therefore, all aerobes have had to develop defenses to resist or repair the damaging effects of ROS over the course of the 1.5 billion years of obligate endosymbiotic co-evolution. During this time mitochondria have developed a defense system to deal with oxidative stress similar to that of their cellular host (Zhong & Yin, 2015). However, excessive antioxidant defense when aerobes emerged would have limited subsequent evolution by protecting their DNA from mutations (Harman, 1981). There are four subclasses of antioxidant defense systems: (i) primary antioxidant defense (deals directly with ROS): superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT), (ii) additional defenses that support primary antioxidant defense, including metal/protein complexes such as ferritin, transferrin, ceruloplasmin, metallothionein and low molecular weight antioxidants such as ascorbate, melanin, melatonin and uric acid, (iii) nonenzymatic dietary antioxidants such as vitamin E, carotenoids and plant polyphenols and (iv) enzymes that repair biomolecules damaged by ROS (Storey, 2004). For example, there are three major detoxification pathways to convert the lipid peroxidation (LPO) product, 4-HNE to less reactive chemical species: aldo-keto reductases (AKRs) or alcohol dehydrogenases (ADH) and aldehyde dehydrogenase (ALDH). AKRs and ADH are present in mitochondria. ALDH2, one member of the ALDH family, is exclusively located in mitochondria. (Zhong & Yin, 2015). Manganese-dependent superoxide dismutase (MnSOD, SOD2) converts superoxide anion into H_2O_2. This itself can lead to the generation of reactive species which can be damaging to lipids, DNA and proteins. Glutathione (GSH) serves as a buffer to minimize creation of harmful molecules and is one of the most important hydrophilic antioxidants in mitochondria (Hosamani & Muralidhara, 2013; Zhong & Yin, 2015). Glutathionylation has a key role in detoxifying mitochondria from the H_2O_2 produced by the RC under conditions where complex III is interrupted, thus protecting against the generation of oxidative stress (Garcia-Ruiz *et al.*, 1995). One frequently employed mechanism to decrease mtROS production is to increase the rate of metabolic uncoupling. The

uncoupler-like proteins UCP2 and UCP3, located in the IMM, have been found to serve as proton channels through which protons pass from the intermembrane space to the mitochondrial matrix. This results in dissipation of the proton gradient across the membrane, and partial conversion of the membrane potential into heat. Uncoupling proteins (UCPs), notably the canonical UCP1, become abundant in mitochondria of animal tissues during prolonged cold periods, and are also present in heat-producing mitochondria of brown fat. UCP2 and UCP3 are proposed to decrease ROS emission from mitochondria (Guzman *et al.*, 2010; Toime & Brand, 2010; Mailloux *et al.*, 2011).

ROS defense can be compromised by either genetic mutations which impair the activity of the antioxidants, e.g., familial cases of amyotrophic lateral sclerosis (ALS) with a mutation in CuZn SOD (SOD1, Mariani *et al.*, 2005), or by increased radical production (Trushina & McMurray, 2007). In both cases oxidative stress will damage cellular function (Hamran, 1956). Such damage is also associated with a number of age-related diseases, including atherosclerosis, arthritis, muscular dystrophy, macular degeneration, insulin resistance associated with type 2 diabetes, pulmonary dysfunction, various neurological disorders, different types of cancers, cardiovascular diseases, and psychiatric diseases, including depression, autism and schizophrenia (Brieger *et al.*, 2012). Under pathophysiological conditions mtROS can initiate and promote cancer but can also serve as an Achilles' heel in tumor therapies (Sabharwal & Schumacker, 2014). mtROS can also initiate the production of Aβ (the beta amyloid peptide implicated in Alzheimer's disease) *in vitro* and *in vivo* (Leuner *et al.*, 2012) and aging itself is associated with long term exposure to oxidative stress (Harman, 1956). However, there are reports that oppose the association between increased ROS and aging and this seems paradoxical. A lifespan-promoting role of ROS has been termed as mitohormesis and is believed to be behind increased lifespan mediated by caloric restriction (Ristow & Zarse, 2010). Hormesis is a "process in which a low dose of a chemical agent or environmental factor that is damaging at higher doses induces an adaptive beneficial effect on the cell or organism" (Mattson *et al.*, 2008). The generation of ROS also occurs through exposure to numerous exogenous agents and biological events including ionizing radiation, UV, cytokines, growth factors, chemotherapeutic drugs, environmental toxins and hyperthermia (Salmon *et al.*, 2007).

Changes in the cellular redox equilibrium may also influence developmental pathways in a variety of tissues from phylogenetically diverse organisms (Sohal *et al.*, 1996). Increased ROS generation plays a role in cell differentiation (and a decrease in protein glutathionylation) (Sohal & Allen, 1986; Sohal *et al.*, 1996). SOD activity

increases during metamorphosis of the fruit fly *Ceradtitis capitatae* (Fernandez-Souza & Michelson, 1976). MnSOD activity increases during neonatal rat brain development (Mavelli *et al.*, 1982) and during monocyte transformation into macrophages in humans (Nakagawara *et al.*, 1981). ROS can act as secondary messengers in cellular pathways which protect against, or repair damage (Ristow & Schmeisser, 2011, Yee *et al.*, 2014). Increased mtROS in the cell is believed to induce an adaptive reaction, e.g., the mitochondrial unfolded protein response (UPRmt) which leads to resistance to stress and eventually decreases oxidative stress (Yoneda *et al.*, 2004; Runkel *et al.*, 2013; Nargund *et al.*, 2015).

Ferroptosis is a form of regulated cell death driven by iron-dependent lipid peroxidation with preferential oxidation of phosphatidylethanolamine (Kagan *et al.*, 2017). Loss of lipid repair enzyme, gluthatione peroxidase 4 (GPX4), which reduces hydroperoxides of polyunsaturated fatty acids (PUFA) and phospholipids (Imai & Nakagawa, 2003), leads to accumulation of lipid hydroperoxides and to fatty acid radical generation, causing plasma membrane damage and ferroptotic cell death (Yang *et al.*, 2016; Agmon *et al.*, 2018). Where and how fatty acid radicals are generated is not known (Friedmann Angeli *et al.*, 2014; Gao *et al.*, 2018), but accumulation of lipid hydroperoxides is iron-dependent. Iron chelators are found to block ferroptosis (Dixon *et al.*, 2012), explaining the term chosen for this type of cell death.

It is not known if mitochondria are involved in ferroptosis. What we know is that cells undergoing ferroptosis exibit electron-dense mitochondria and OMM rupture. Both morphological and chemical changes occur in mitochondria and contribute to ferroptosis (Friedmann Angeli *et al.*, 2014). Mitochondrial permeabilization in ferroptosis is not dependent on BAX/BAK and BCL2 which are involved in apoptotic cell death (Dixon *et al.*, 2012). However, when mitochondria are depleted via mitophagy, cells become more resistant to ferroptosis (Gao *et al.*, 2019), demonstrating that the mitochondrion is a crucial player in ferroptosis.

Given that the role of ROS in the cell is complex, the redox effects of cellular manipulations are context- and organism-dependent.

2.2 *Drosophila* development and its regulation

Metazoan development involves the coordinated activity of signaling pathways to regulate gene expression. This controls and executes the program of cell division, differentiation and cell deaths that shape the animal and its component parts, as it

matures. *Drosophila* is one of the classic model organisms in which this process has been studied.

The *Drosophila* life cycle comprises embryogenesis, three larval stages, a pupal stage, and the adult stage. Embryogenesis occurs during the first day of the life cycle (after egg laying), and it involves segmentation, gastrulation and organogenesis. During development, a majority of animals first develop small versions of their adult body structures, followed by size increase. The development of *Drosophila* and of many insects as a whole differs. Embryogenesis gives rise to the larva, which represents a highly modified and typically simplified version of the adult body plan. During the larval stages, precursor structures are set aside as imaginal discs, that will develop later into the adult body components. Clusters of imaginal disc precursor cells invaginate from the embryonic epithelium and form these structures during larval development. The embryonic imaginal-disc precursor cells are in a G0-like quiescent state, but start dividing rapidly during larval growth (Duronio, 1999). Larvae feed and increase cell mass to the point where this triggers reactivation of the cell cycle (Edgar & Lehner, 1996). These precursors give rise to the imaginal discs, which are protected in the larva but do not develop further until the pupal stage, when they do not simply grow, but will be greatly transformed during metamorphosis. Imaginal discs consist of a single-layered epithelium adjoining the epithelium of the larval epidermis (Poodry & Schneiderman, 1970). If imaginal discs are dissociated, the integrity of the epithelium is destroyed (Poodry *et al.*, 1971). There are 20 imaginal discs which develop their internal pattern as the larva grows. At metamorphosis, they evert (turn inside out), extend, and differentiate to form the epidermal layer of the adult fly body, including its appendages (wings, legs, eyes, halteres, and genitals). To form the thorax of the adult, migrating cells from pairs of contralateral discs eventually meet and fuse, joining also to the adjacent ipsilateral discs to form a continuous epithelium (Poodry *et al.*, 1971). Cell identity within imaginal discs is controlled by position along the major body axes, e.g., anterior/posterior. Decapentaplegic (Dpp) and Wingless (Wg) are morphogens that pattern cell types along the dorsal/ventral and anterior/posterior axes, respectively, and are involved in growth and proliferation of imaginal cells (Duronio, 1999). In addition to the discs, other imaginal structures include histoblast nests, which will form the abdominal epidermis, and small groups of cells that will give rise to the gut or salivary glands (Cohen *et al.*, 1993).

Postembryonic development is controlled by fluctuations in the level of the steroid hormone 20-hydroxyecdysone (20E), traditionally called ecdysone. These are known as ecdysone pulses. Each pulse causes different developmental events by

activating stage- and tissue-specific gene expression programs (Riddiford, 1993). During the early stages of metamorphosis, most of the larval tissues go through programmed cell death in a process termed histolysis. Histolysis is followed by developing new structures through morphogenesis and differentiation that will make an adult fly (Baehrecke, 1996). Post-embryonically, larvae mature and grow using two modes of cell-cycle progression: the canonical G1-S-G2-M cell division cycle and the endoreduplication, or 'endo', cycle. Larval tissues that will not contribute to adult structures typically grow via the 'endo', cycle where DNA is replicated repeatedly without nuclear or cell division (Duronio, 1999).

2.2.1 Signaling pathways regulating growth and cell migration during development

To shape the structures of the organism, different cells initiate or cease proliferation at defined times during development. Mechanisms that coordinate growth, patterning, and cell proliferation in developing tissues are evolutionarily conserved and regulated by many of the same signaling pathways in vertebrates and *Drosophila* (Edgar & Lehner,1996), e.g., during mid-embryogenesis. These signaling pathways are highly interconnected, creating a vast diversity of cellular responses that are executed by relatively few signaling pathways: NF-κB Wingless/Wnt, receptor tyrosine kinase/extracellular regulated kinase (RTK/ERK), Jak-STAT, Akt/Tor, Notch, TGF-β, G protein coupled receptors (GPCR), Hedgehog, Toll, and steroid hormone pathways (Friedman & Perrimon, 2007). Output from the downstream transduction network will depend on the context, and the intensity and duration of signaling (Housden & Perrimon, 2014). For simplicity, I will briefly introduce some of the developmental pathways of the fly, which is the model organism used in most of my study, focusing on aspects that are important for the discussion of this thesis. I will also refer, in passing, to relevant studies on other organisms.

Receptor tyrosine kinase (RTK) signaling has many functions during development. Particularly important are RTKs activated by fibroblast growth factors (FGFs), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and glial cell line-derived growth factor (GDNF). Signaling malfunctions in these pathways causes a range of developmental disorders (Basson, 2012) in humans, including cancer.

The mitogen-activated protein kinase (MAPK) cascades are central signaling pathways that regulate proliferation, differentiation, apoptosis, stress responses, cell

migration and survival. Each cascade consists of three core protein kinases: MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAPKK), and MAPK, additionally accompanied by upstream MAP kinase kinase kinase kinase (MAP4K) and downstream MAP kinase-activated protein kinase (MAPKAPK) components. Within each of the cascades, the signal is propagated by phosphorylation and activation of the sequential kinases, eventually leading to the phosphorylation of target regulatory proteins by the MAPK and MAPKAPK components (Flores *et al*., 2018). There are four different mammalian MAPK cascades named according to their MAPK components, as follows: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, and ERK5 (Kyriakis & Avruch, 2001). After stimulation, the MAPK, as well as the MAPKAPK components phosphorylate different substrates in many cellular locations, and these are responsible for initiating diverse cellular processes, such as proliferation, differentiation, fate determination, neuronal plasticity, survival, and under some conditions stress responses and apoptosis (Yoon & Seger, 2006; Plotnikov *et al*., 2011). The JNK subgroup of MAPKs have been implicated in morphogenetic events in both mouse and *Drosophila* (Pai *et al*., 2012) development.

Steroid hormones work by binding to nuclear receptors, which activate transcription in a ligand-dependent manner (Bai *et al*., 2000). They have a role in stimulating invasive cell behavior, independent of effects on proliferation, as well as many other physiological and developmental processes. Dysregulation of steroid hormones or of nuclear receptor signaling in general, are major underlying factors in diseases such as metastasis of breast and ovarian cancer, because of effects on cell migration (Anzick *et al*., 1997; Bai *et al*., 2000). The maturation process in both insects and vertebrates is triggered by rises in hormone titres, and transduced by members of the nuclear-receptor superfamily. These functions are primarily controlled by thyroid hormone and sex steroids in vertebrates, whereas in holometabolous insects such as *Drosophila melanogaster*, genetic regulation of maturation occurs during metamorphosis and is triggered by rises in the ecdysone. (King-Jones & Thummel, 2005). The ecdysone receptor is a heterodimer composed of Ultraspiracle (Usp) and the Ecdysone receptor (EcR). Usp is a member of the nuclear hormone receptor superfamily, considered as the fly retinoid X receptor (RXR) homolog. Amongst many roles, ecdysone regulates border cell migration. Border cells are groups of follicle cells in the *Drosophila* ovary and are a model system to the study cell motility (King, 1970; Montell, 1999). Border cells lacking Usp are unable to migrate (Oro *et al*., 1992) and defects in border cell migration lead to a failure of fertilization (Montell

et al. 1992). Recessive lethal *usp* mutations exhibit a heterozygous phenotype of cleft thorax (Henrich *et al.*, 1994).

Cleft thorax is a midline closure defect arising from a failure of cell migration, affecting the thorax and/or scutellum, the latter being a shield-like plate at the posterior of the mesothoracic tergum. Depending on its causes, the severity of clefting can range from mild to severe. The cleft may arise either from the improper fusion of the discs along the dorsal midline of the notum, or a mutant effect on cells lying along the midline of the mesothoracic disc. Affected flies also exhibit bent and misshapen sensory bristles and severely enlarged legs (Henrich, 1994).

AMP-activated protein kinase (AMPK) is best known as a regulator of cell metabolism, maintaining the balance between ATP production and consumption in all eukaryotic cells because it senses cellular energy levels (Hardie, 2007). In addition, AMPK maintains polarity in epithelial cells (Hardie, 2007) and regulates cell migration by controlling microtubule (Nakano *et al.*, 2010) and actin cytoskeleton dynamics and reorganization at the plasma membrane (Bae *et al.*, 2011; Kondratowicz *et al.*, 2013). AMPK activators are currently in clinical use to inhibit cell migration. However, the underlying mechanism is unknown (Yan *et al.*, 2015).

2.3 Cell migration

Anthony van Leeuwenhoek was the first to observe and report cell movement in 1674 (see Risler, 2009). Cell migration is necessary for the processes in animal development that create the 3D forms of tissues, organs and the whole body, described as morphogenesis. Many such processes take place during embryonic development, which shape the emerging organism (Stossel, 1993). Cell migration is also important in skin and intestine renewal and immune system function. Fibroblast and vascular endothelial cell migration is essential for wound healing (Lauffenburger & Horwitz, 1996; Montell, 1999). The ability of human spermatozoa to migrate is instrumental in fertility (Denissenko *et al.*, 2012). After mammalian fertilization, selected cells of the developing embryo migrate to the uterine wall to establish the placenta (reviewed by Stossel, 1993).

Misregulation of cell migration is implicated in diseases such as birth defects, cancer metastasis, mental retardation, atherosclerosis, osteoporosis and arthritis (Montell, 1999; Davis *et al.*, 2003). It remains a major question in biology to fully understand how cells move, or to use Stossel's (1993) terminology, how do cells crawl, when do they start their movement and what are the stop signals, once the

destination has been reached. To migrate, cells need to be able to polarize and extend two protrusive structures: membrane extensions on the leading edge of the cell called lamellipodia and actin-rich plasma-membrane microspikes that extend beyond the leading edge of lamellipodia in migrating cells and serve as antennae for cells to probe their environment, called filopodia (Ridley *et al.*, 2003; reviewed in Mattila & Lappalainen, 2008). Protrusions are driven by actin polymerization, and adhere to the extracellular matrix or nearby cells via transmembrane receptors (Ananthakrishnan & Ehrlicher, 2007). By protruding in this way, the cell makes a traction site towards the migration point and releases the traction from the rear end. Protrusions are made by membrane movements which are believed to occur by an 'elastic Brownian ratchet' mechanism, in which thermal energy bends the nascent short filaments, storing elastic energy (Mogilner & Oster, 1996; Mogilner & Oster, 2003; Ananthakrishnan *et al.*, 2007). At a point in time when the plasma membrane (PM) is most distant from the filament end, a new monomer of actin can be added. Consequently, the PM is no longer able to return to its former position since the filament is now longer. The filament cannot be pushed backwards by the returning PM as it is locked into the mass of the cell cortex by actin binding proteins. In this way, the PM is permitted to diffuse only in an outward direction (Ridley *et al.*, 2003; Maciver, 2001). Cell migration is a cyclic process of protrusion, adhesion to the substrate, contraction of the cell body, and rear retraction (Abercrombie *et al.*, 1971). Collective migration is defined as the ability of groups of cells to move together while tightly connected and influencing one another on their way (Rørth, 2012; Theveneau & Mayor, 2012). Both epithelial and mesenchymal cells can migrate collectively and interact with their extracellular environment during migration. Leader cells express different genes compared with internal and follower cells (Rørth, 2012). Epithelial cells maintain cell-cell adhesions. Leader cells form protrusions oriented in the direction of migration, whereas followers form smaller cryptic protrusions. Mesenchymal cells migrate directionally as a collective, but they form only transient cell-cell connections. Epiboly, neural tube closure and embryonic wound healing in vertebrates, ventral enclosure in *Caenorhabditis elegans*, and dorsal closure of the *Drosophila* embryo are morphogenetic movements that occur because of epithelial cell sheets being able to collectively migrate, spread and fuse (Bard, 1990).

2.3.1 Cell migration in *Drosophila*; single cell migration and epithelial sheet movement

During embryogenesis of the fruit fly a complex cell migration takes place. In the embryo, migrating cells are: primordial germ cells (PGCs), cells of the tracheal system, hemocytes, the fly's phagocytic cells (Montell, 1999), and border cells (King, 1970). Border cell migration resembles the migratory behavior of human ovarian cancer cells, serving as a model to study molecules that promote spreading of human ovarian cancer (Yoshida *et al.*, 2004). Neuronal and glial cells go through limited cell migration to establish axonal pathways (Klambt *et al.*, 1991). Gastrulation involves massive cellular movements of the germ layers (Leptin, 2005), germ band retraction and dorsal closure, described in more detail in the next sub-section. The mechanisms that decide which cells become migratory and how cells physically move are poorly understood.

2.4 *Drosophila* dorsal closure and thorax closure; JNK signaling role

Dorsal closure (DC) is one of the most studied processes involving epithelial movement and fusion in metazoans, and has been employed as a model system to study wound healing (Harden, 2002) and movement of epithelial cell layers in general (Knust, 1997).

DC begins in the middle of embryogenesis and lasts approximately two hours (Campos-Ortega & Hartenstein, 1985). The DC process goes through three phases (Noselli, 1998). First, dorsally located epidermal cells, leading-edge (LE) cells, elongate along the dorsoventral axis (Fig. 1 A). JNK signaling activation is restricted to LE cells during DC (Noselli, 1998). JNK activates the morphogen Dpp in the LE which controls the migration of the more lateral cells (Fig. 1 B). Third, LE cells from both sides meet on the dorsal surface and form the midline (Knust, 1996) (Fig. 1 C). DC relies exclusively on cell elongation. There is no cell proliferation or recruitment in this process (Young *et al.*, 1993).

DC is supported by apoptosis in the amnioserosa (Toyama *et al.*, 2008). Apoptosis provides part of the force which drives DC (Toyama *et al.*, 2008) and gives mechanical tension in wound healing which helps tissue reconstruction (Rosenblatt *et al.*, 2011).

There are mutations which disturb different stages of DC (Knust, 1996). Mutants with defects in DC are described as 'dorsal open' because the mutant phenotype resembles a hole on the dorsal surface which reflects the failure of the movement of the lateral ectoderm. Mutations in core JNK signaling genes, DJNKK gene *hemipterous* (*hep*, JNKK, Glise *et al.* 1995) and DJNK *basket* (*bsk*, JNK, Riesgo-Escovar *et al.*, 1996) disrupt DC because LE cells cannot form filopodial and lamellipodial protrusions. The core signaling module of JNK signaling consists of JNKK (Hep) which when activated phosphorylates JNK (Bsk) (Glise *et al.*, 1995), which gets activated and phosphorylates Jun (Jra) (Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996) and Fos (Ciapponi *et al.*, 2001). Jun (Jra), together with Fos (Kay) form the AP-1 transcription factor which then activates JNK target genes and contributes to expression of TGF-β family protein Dpp in the leading edge of epithelial cells (Hou *et al.*, 1997). Dpp then contributes to elongating the lateral edge of the embryo. During DC, the JNK signaling pathway is activated in the LE by an unknown signal. Another known target of JNK is *Drosophila* MAPK phosphatase Puc (Martín-Blanco *et al.*, 1998). Puc controls JNK signaling in the LE (Martín-Blanco *et al.*, 1998) by negative feedback, which is important to stop cell movement at the end of the process (Noselli & Agnès, 1999). In addition to specific mutants in JNK signaling that exhibit a dorsal-open phenotype and fail to complete embryogenesis, others do not complete disc eversion or are not able to fuse discs during metamorphosis (Pastor-Pareja *et al.*, 2004).

Apart from the JNK signaling pathway, there are other genes which can be mutated to generate the dorsal-open phenotype. These encode proteins involved in a variety of cellular processes, including components of the cytoskeleton (e.g., zipper), membrane proteins (coracle, yurt), cell adhesion proteins (shotgun, armadillo, myosheroid, scab) and extracellular matrix proteins such as pericardin (Simonova & Burdina, 2009).

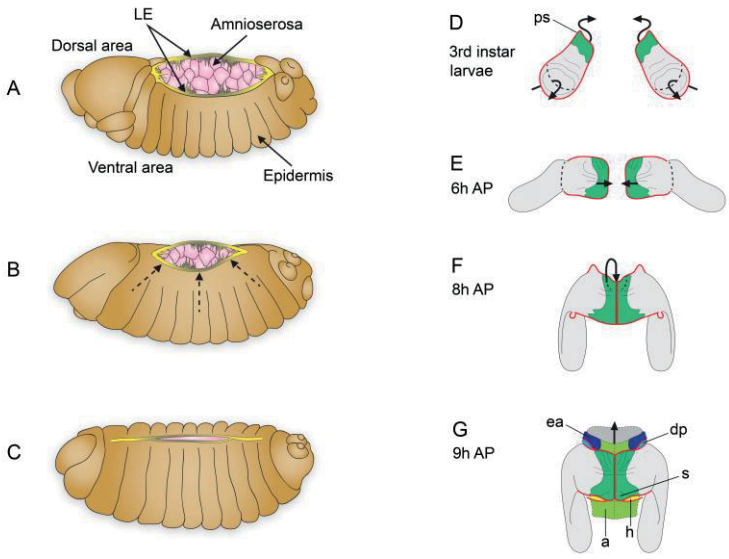


Figure 1. Dorsal and thorax closure in *Drosophila*. (A-C) Dorsal closure. (A) At embryonic stage 13, the epidermis covers the ventral and lateral sides of the embryo, while the amnioserosa (pink) covers the dorsal side. LE- leading edge (yellow). (A, B) Filopodial and lamellipodial protrusions on LE cells (yellow) during DC (Jacinto *et al*., 2000). (B) During the second stage, the epidermis moves dorsalward (dashed arrows), followed by elongation of the cells in the LE and lateral cells. (C) At the end of DC (embryonic stage 15), the two LE (yellow) meet at the dorsal midline and the cells fuse. (D-G) Dorsal thorax development. (D) The cells at the margin of the larval wing disc proper (red) will attach to other imaginal discs and form the continuous adult epidermis. (E-G). (D) In third instar larvae, the wing imaginal disc is attached to the larval body wall by the peripodial stalk (ps). During pupariation the disc epithelium everts through the widened peripodial stalk (upper arrow) and spreads inside the pupal case to replace the larval epidermis. At the same time, the wing blade develops through invagination of the disc epithelium (lower arrow). (E) At 6 hours after puparation (AP), the dorsal parts approach each other (arrows) and attach to each other. The disc margin on the ventral side (dashed line) attaches to the leg discs to complete the ring-like structure of the thorax complex. (F) At around 8 hours AP, the anterior part of the future notum folds inside (arrow and dashed line), presumably to attach to the eye-antennal imaginal disc before it moves anteriorly. (G) At 9 hours AP, during head eversion (arrow), the wing imaginal disc is attached to all its neighbor imaginal discs: anteriorly to the eyeantennal disc (ea) and the dorsal prothoracic disc (dp), and posteriorly to the abdomen (a) and haltere disc (h). The border between them is marked in red. The final shape of the notum is obtained with further tissue movements. The scutellum (s) presumably forms by a dorsoposterior protrusion. Starting during pupariation dorsal parts

of two imaginal wing discs migrate until they finally attach to each other (black arrows). The expression domain of *pannier* (*pnr*, green) marks the dorsalmost stripe of the future adult epidermis. The *pnr*^{MD237} GAL4 driver used in the experimental work of this thesis has the same expression pattern. Modified from Zeitlinger & Bohmann (1999); Simonova & Burdina (2009).

Along with JNK- and dpp-signaling, the Notch signaling pathway regulates DC. Notch mutants may partially restore the phenotype caused by the abnormalities of the JNK pathway (Zecchini *et al.*, 1999). It is believed that the transmembrane receptor Notch negatively controls one of the probable alternative signaling pathways (probably, MAPK), which is able to partially compensate for the loss of JNK signal in relevant mutants.

2.4.1 Differences between dorsal and thorax closure

Thorax closure starts at the anterior end of the wing disc, proceeds through the most posterior region, and, finally, fills the gap (Martín-Blanco *et al.*, 2000). JNK and dpp signaling mechanisms during thorax closure resemble those involved in DC, but with some differences (Zeitlinger & Bohmann, 1999; Martín-Blanco *et al.*, 2000; Pastor-Pareja *et al.*, 2004). Imaginal cells are brought together by spreading over the larval epidermis in a process mediated by the extension of filopodia, which seem to pull the contralateral epithelial sheets toward the midline. JNK signaling is involved in both cell spreading and imaginal disc fusion (Martín -Blanco *et al.*, 2000), while Dpp participates in the regulation of the actin cytoskeleton of cells at the imaginal LE and the maintenance of cell polarity (Ricos *et al.*, 1999). However, there are some differences between dorsal and thorax closure illustrated in Fig. 2. During embryonic DC, the amnioserosa and the epidermal cells maintain their relative positions and, despite occasional delaminations, amnioserosa cells remain in place until the very end of the process. They detach from the overlying epidermis only when closure is completed, and undergo apoptosis. During disc spreading that leads to thoracic closure, imaginal cells crawl over the larval epidermis (Fig. 2). In this process, larval cells are left below and behind and eventually delaminate from the edges (Martín-Blanco *et al.*, 2000).

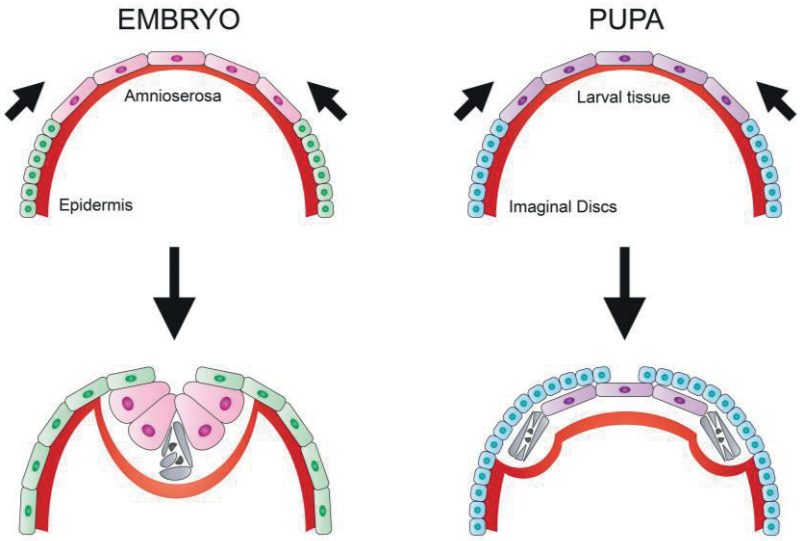


Figure 2. Dorsal and thorax closure differences. Embryonic DC starts with the stretching of epidermal cells (green) and the simultaneous contraction of the apical ends of amnioserosa cells (pink). Amnioserosa and epidermis remain continuous during the whole process remaining in contact with the basal lamina (red). Once DC is finished, amnioserosa cells delaminate and undergo apoptosis (gray). During imaginal thorax closure, imaginal cells (blue) detach from the basal lamina (red) and using filopodia crawl over the larval cells (purple). Here, imaginal cells leave the larval cells behind and larval cells subsequently delaminate from the borders of the larval sheet and undergo apoptosis (gray). Modified from Martín-Blanco (2000).

2.5 JNK signaling in mammals; parallels with the *Drosophila*

MAPKs are crucial regulators of cell migration and the morphogenetic movement of epithelial sheets (Xia & Karin, 2004). JNK signaling pathway is evolutionary conserved and it is not surprising that apart from being involved in *Drosophila* dorsal and thorax closure it is also involved in mice in closure of the eyelid, neural tube (Sabapathy *et al.*, 1999; Kuan *et al.*, 1999) and optic fissure (Weston *et al.*, 2003). Knockout mice for c-Jun have defects in eyelid closure and in fibroblast migration during wound healing (Grose, 2003; Javelaud *et al.*, 2003; Li *et al.*, 2003; Zenz *et al.*, 2003; Weston *et al.*, 2004). c-Jun is essential for embryonic development, as fetuses lacking the protein die at mid-gestation with impaired hepatogenesis (Hilberg *et al.*, 1993). In rodent embryos, following cuts to the skin, *c-fos* and *transforming growth factor*

beta (TGFβ-1) which are homologs of *Drosophila kayak* and *dpp* are activated at the edge of the wound (Martin & Nobes, 1992).

In vertebrates a phenomenon similar to DC occurs, described as embryonic wound healing. In contrast to mature individuals, this process does not result in scar formation (Martin *et al.*, 1993; Martin, 1997). After a mouse or rat embryo's skin is injured both c-Fos and TGFβ-1 are activated on the edge of the wound. They are the homologs of the *D. melanogaster* genes *kayak* and *dpp*, respectively (Martin & Nobes, 1992).

Primary c-Jun-/- fibroblasts have a severe proliferation defect and undergo premature senescence *in vitro* (Johnson *et al.*, 1993; MacLaren *et al.*, 2004). The mammalian AP-1 transcription factor regulates the expression of many genes, such as those encoding matrix metalloproteinases (MMPs), integrins, cytokines, and growth factors (Angel *et al.*, 2001, Yates & Rayner, 2002; Florin *et al.*, 2004), which are involved in wound healing. The Scribble (Scrib) complex contributes to epithelial cell migration and fusion during both development and wound healing in mammals by recruiting the small GTPases Cdc42 and Rac and the serine/threonine kinase Pak to the LE of migrating cells (Murdoch *et al.*, 2003; Zarbalis *et al.*, 2004; Humbert *et al.*, 2006; Dow *et al.*, 2007; Bahri *et al.*, 2010). The JNK-dependent network of transcriptional factors also contributes towards epithelial to mesenchymal transition (EMT), a biological process in which cells lose cell-cell adhesion and become migratory (Sahu *et al.*, 2015). JNK and the Wnt pathway are main regulators of wound repair and tissue regeneration. In wound healing, the actin cytoskeleton (Hall, 1998; Kaibuchi *et al.*, 1999) is a major JNK target, as well as growth factors. Mechanical stretching of the wound-edge cells, loss of cell polarity or necrotic signals are reported to be upstream activators of this signaling pathway (Rämet *et al.*, 2002; Nelson *et al.*, 2005; Igaki *et al.*, 2006). Expression of Wnt target genes is also stimulated by JNK activation to accelerate tumorigenesis (Sancho *et al.*, 2009).

Developmental morphogenesis, tissue injury, and oncogenic transformation can cause the detachment and elimination of epithelial cells by anoikis, a specialized form of apoptosis (Girnius & Davis, 2017). Loss of JNK function by epithelial cells may lead to survival of these cells in luminal spaces and if these cells accumulate additional mutations that may cause cancer. JNK deficiency accordingly enhances tumor formation in a model of breast cancer (She *et al.*, 2002; Cellurale *et al.*, 2012; Hübner *et al.*, 2012). Two upstream components of the JNK pathway (MAP2K4 and MAP3K1) are frequently mutated in human cancer (Stephens *et al.*, 2012, Nik-Zainal *et al.*, 2016). However, an overactive JNK pathway also has been shown to cause cancer (Smeal *et al.*, 1991; Hui *et al.*, 2008; Barbarulo *et al.*, 2013). This means that the

JNK pathway performs opposing functions in oncogenic signaling. Downregulation of JNK signaling leads to developmental defects, whilst an overactive JNK pathway can lead to cancers (Bubici & Papa, 2014). Abnormal JNK signaling contributes to many pathological conditions such as stroke (Wu *et al.*, 2000; Borsello *et al.*, 2003), obesity and insulin resistance (Hirosumi *et al.*, 2002), and atherosclerosis (Ricci *et al.*, 2004). JNK activity has also been implicated in Parkinson's and Alzheimer's diseases (Zhu *et al.* 2001; Peng & Andersen, 2003), whilst JNK function is needed for brain development, memory and learning (Raivich & Behrens 2006; Waetzig *et al.* 2006).

2.5.1 Interplay between JNK signaling and mitochondrial metabolism

There is a multilayered link between JNK signaling and mitochondria. JNK is a major signaling pathway regulating both metabolic (Harris *et al.* 2002) and apoptotic (Kharbanda *et al.* 2000; Schroeter *et al.*, 2003) functions in mitochondria, linking cytosolic and mitochondrial energy pathways. For example, it has been shown that, in primary cortical neurons, JNK-induced decline in pyruvate dehydrogenase (PDH) activity resulted in the decline of ATP levels and increased lactate concentration (Zhou *et al.*, 2008). These observations suggest a shift from aerobic metabolism of pyruvate in mitochondria to its anaerobic reduction to lactate in the cytosol with concomitant NAD^+ regeneration to support glycolysis. JNK may therefore serve as an important modulator of mitochondrial bioenergetics, anaerobic respiration and cellular ATP levels, regulating mitochondrial electron flow as well as the production of ROS from the RC (Zhou *et al.*, 2008). JNK signaling is associated with the regulation of mitochondrial pathways of apoptosis following viral infection. After reovirus infection, MEKK1 activates JNK, which promotes the release of the proapoptotic factors Smac and cytochrome *c* (Cyt *c*) from mitochondria, both of which contribute to apoptosis (Clarke *et al.*, 2004). Mitochondrial H_2O_2 has been shown to activate JNK in various cell lines upon inhibition of MAPK phosphatase or dissociation of the glutathione transferase-JNK complex (Nemoto *et al.*, 2000; Chen *et al.*, 2001; Foley *et al.*, 2004). Prolonged JNK activation is believed to play a key role in cell injury. For example, apoptosis of auditory neurons as a consequence of oxidative damage has been shown to engage the major downstream target of the JNK signaling cascade, i.e., c-Jun (Scarpidis *et al.*, 2003). This can be prevented by administration of an inhibitory peptide targeted against JNK (Eshraghi *et al.*, 2006). Mitochondria produce H_2O_2 and NO (Cadenas & Davies, 2000; Poderoso *et al.*, 2000), which are involved in the regulation of redox-sensitive cell signaling through

the JNK pathway, thus coordinating functional responses between mitochondria and other cellular processes. For example, H_2O_2 activates JNK at multiple levels by mechanisms eliciting dissociation of inhibitory complexes (Saitoh *et al.*, 1998) or suppression of phosphatases involved in kinase inactivation (Chen *et al.*, 2001). Nitric oxide inhibits JNK activity by S-nitrosylation of a critical cysteine thiol (Park *et al.*, 2000). In contrast, JNK as a signaling molecule translocates to mitochondria under stress conditions (Kharbanda *et al.*, 1997; Aoki *et al.*, 2002) and has direct metabolic effects in mitochondria, through the activation of phosphorylation cascades (Zhou *et al.*, 2008). This network thus connects cytosolic and mitochondrial processes that control energy levels and the redox environment throughout the cell (Zhou *et al.*, 2008).

2.6 Alternative oxidase: proposed roles in living organisms

The alternative mitochondrial respiratory chain contains the alternative NADH dehydrogenases (NDX) and quinone oxidases (AOX) (McDonald &Gospodaryov, 2018). AOX, which is present in most organisms, is a non-proton motive ubiquinol oxidase (Berthold & Stenmark, 2003), i.e. it catalyzes the oxidation of ubiquinol and consequent reduction of oxygen to water (McDonald & Gospodaryov, 2018), and is a branch point in the respiratory electron transport chain, bypassing complexes III and IV (McDonald *et al.*, 2009) (Fig. 4). By virtue of this property it dramatically decreases ATP production and released energy is dissipated as heat (Rhoads & Subbaiah, 2007). AOX is a membrane bound diiron carboxylate enzyme (Siedow *et al.*, 1995). Depending on species it can occur as a monomer, homodimer or even multimer, with its iron atoms coordinated by glutamic acid residues (Berthold & Stenmark, 2003). AOX activity depends on the membrane-bound concentration and redox state of ubiquinone, as well as cellular oxygen concentration, and is regulated via gene expression or by post-translational modification. In the latter case, cellular redox status affects tertiary complex formation, whilst enzymatic activity is modulated by the action of allosteric effectors such as pyruvate or other metabolites. The ratio of oxidized to reduced protein varies considerably between species and tissues. In plant mitochondria, metabolites such as pyruvate, hydroxypyruvate and glyoxylate can activate AOX (Sluse & Jarmuszkiewicz, 1998).

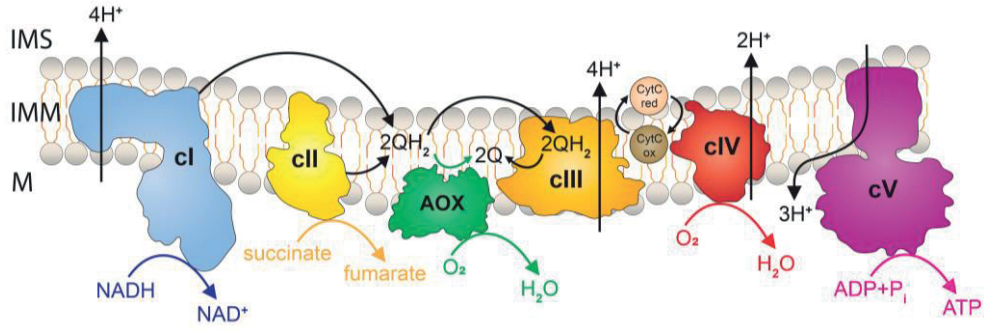


Figure 3. Mitochondrial respiratory electron transport chain showing AOX, which is a branch point at the level of ubiquinol. Electron flow from ubiquinol to complex IV (reducing O_2 to H_2O) is coupled to proton transport (at two sites) while electron flow from ubiquinol to AOX (reducing O_2 to H_2O) is not coupled to proton transport. Complexes I, III and IV pump protons (H^+) across the IMM to the IMS, generating a proton gradient which is used by complex V to produce ATP. IMS, intermembrane space; IMM, inner mitochondrial membrane; M, mitochondrial matrix; cI-cV, complexes I-V; QH_2, ubiquinol (dihydroxyquinone); Q, ubiquinone (quinone); Cyt *c* red, cytochrome *c* reduced; Cyt *c* ox, cytochrome *c* oxidized.

The fungal AOX is stimulated by purine nucleotides, specifically ADP, AMP and GMP (Affourtit *et al.*, 2002). However, any post-translational regulation as well as its activation and inhibition kinetics of AOX expressed in animals is unknown (Tward *et al.*, 2019). In a number of organisms, the synthesis of AOX protein can be induced by superoxide anion (Minagawa *et al.*, 1992). AOX mRNA and AOX activity can also be induced by H_2O_2 (Vanlerberghe & Mclntosh, 1996). All residues necessary for formation of the AOX's active site and catalytic act are present in the coding sequence, therefore AOX may theoretically function as a monomer. Indeed, there are reports that AOX actually works as a monomer (Shiba *et al.*, 2013) as well as a homodimer (Shiba *et al.*, 2012).

AOX activity is typically determined using tissue, cells, or isolated mitochondria in which oxygen consumption is measured in the presence/absence of AOX and COX inhibitors. Although this approach does not measure activity *in vivo*, it does indicate the capacity of the AOX pathway (Ribas-Carbo *et al.*, 1995). However, oxygen isotope discrimination techniques can accurately measure AOX activity by measuring the flux of electrons through AOX *in vivo* (Robinson *et al.*, 1995; Watling *et al.*, 2006).

The atmosphere of the early Earth is considered to be anaerobic. The first energy-generating reactions presumably involved the breakdown of organic molecules in the

absence of oxygen. Since oxygen is a substrate in the formation of toxic reactive species, it is thought that changes in oxygen levels might have caused selective pressure for microorganisms to develop alternative respiratory proteins (McDonald & Gospodaryov, 2018) that are less vulnerable to ROS damage than other oxidoreductases. Many AOX-expressing organisms are subjected to regular changes in oxygen levels (anoxia/reoxygenation), e.g., plant roots that can be overwatered, causing anoxia (Veiga *et al.*, 2000), or sea squirts that experience functional hypoxia (Torre *et al.*, 2014). In addition, many of them are subjected to conditions which compromise synthesis of specific prosthetic groups of respiratory complexes, notably haem and iron-sulphur clusters, which are susceptible to damage by ROS. Since ROS, generated by NADPH oxidases, are frequently used by immune cells to attack pathogens, this may be one reason why many eukaryotic parasites have retained AOX over the course of their evolution whereas mammals developed different mechanisms to buffer such problems, and have lost the gene for AOX.

AOX genes have so far been found only in slow-moving or sessile organisms (McDonald *et al.*, 2009). However, there are sessile organisms which do not have AOX, suggesting that selective pressure to keep AOX is not only related to the sessile state and low ATP demand, but rather to conditions which compromise function of the respiratory chain. AOX is ubiquitously present in plants, where it is most extensively studied, as well as in many microorganisms, fungi and protists, including human/animal parasites such as *Trypanosoma brucei*, *Cryptosporidium parvum*, *Blastocystis hominis*, and *Candida albicans* (Young *et al.*, 2014). In animals, AOX genes and AOX mRNA have so far been reported in: Placozoa, Porifera, Nematoda, Arthropoda, Mollusca, Annelida, Rotifera, Brachiopoda, Cnidaria, Echinodermata, Hermichordata and Chordata (McDonald *et al.*, 2008; McDonald & Vanlerberghe, 2004; McDonald & Gospodaryov, 2018). It is unclear as to whether the AOX gene is translated into protein in all of these animal species (McDonald *et al.*, 2008). Since AOX is absent in humans and is essential for pathogenic fungi, human parasites and plant pathogenic Nematoda, there is an opportunity for developing species-specific AOX inhibitors which can be tested as potential therapeutics or fungicides (Young *et al.*, 2014). In addition to protection against anoxia, one physiological role of AOX in plants is heat production (Meusse, 1975; Grant *et al.*, 2008; Wang & Zhang, 2015). AOX also has a crucial role in chloroplast protection under extreme environments, such as high light (Xu *et al.*, 2011), as well as tolerance to both biotic and abiotic stress (Saha *et al.*, 2016) especially when resulting in decreased functioning of the cytochrome pathway. Crucially, AOX avoids the generation of excessive ROS resulting from an excess of reducing power accumulated in the ubiquinone pool

(Vanlerberghe, 2013). It therefore enables respiration to resist toxins such as NO, CO, CN^-, etc., thus fine-tuning ATP production, and the maintenance of mitochondrial and cellular homeostasis (Moore *et al.*, 2013).

2.6.1 The role of AOX as gene therapy tool: potential and concerns

RC dysfunction and ROS overproduction are proposed to be instrumental in cancer, aging, neurodegenerative and mitochondrially inherited diseases. By preventing RC blockade and excess ROS production, AOX has therapeutic potential (El-Khoury *et al.*, 2013), and it has been shown that AOX from a tunicate, *Ciona intestinalis*, can be safely and ubiquitously expressed in mammalian cells (Hakkaart *et al.*, 2006), flies (Fernandez-Ayala *et al.*, 2009) and mice (El-Khoury *et al.*, 2013).

AOX could potentially be useful as a gene therapy for diseases caused by mutations in genes which encode subunits of complex III and IV of the respiratory chain and assembly factors for these proteins (Dassa *et al.*, 2009; Kemppainen *et al.*, 2014). In the latter case, AOX would allow electron transfer from ubiquinol to the terminal acceptor (molecular oxygen). Without such transfer, mutant cells would not be able to completely oxidize respiratory substrates for several reasons: (1) product inhibition of upstream enzymes in a biochemical pathway - in effect, the entire pathway becomes blocked, (2) potential toxicity or signaling roles of intermediates within a biochemical pathway, (3) physical laws of membrane energetics - inhibition of the enzymes within the respiratory chain would lead to disturbance in membrane potential. In the case of complex III blockade, ubiquinol would accumulate in the membrane and cause overproduction of superoxide anion-radical by reverse electron transport through complex I. Superoxide anion-radical is able to damage other components of the respiratory chain and other macromolecules in its vicinity. In the case of complex IV blockade, both ubiquinol and reduced cytochrome *c* would accumulate in the respiratory chain. Reduced cytochrome *c* is also a signaling molecule which may exit the mitochondria and trigger apoptosis. If respiratory substrates accumulate (mostly organic acids formed in other catabolic processes, e.g., pyruvate from glycolysis, alpha-ketoglutarate and oxaloacetate - from amino acid transamination, fatty acids and acetyl-CoA from beta-oxidation) and reducing equivalents (NADH) are not oxidized completely, they become toxic to cells. The simplest mechanism of this toxicity is, for instance, lowering of pH, e.g., lactic acidosis. AOX thus has a potential use in diseases where mitochondrial dysfunction is an intermediate step in the pathological process, rather than the underlying cause.

For example, it was shown that expression of *Ciona* AOX mitigates symptoms of Parkinson's and Alzheimer's diseases in *Drosophila* models (Humphrey *et al*., 2012; El-Khoury *et al*., 2016), as well as a lethal inflammatory cascade in a mouse model of sepsis (Mills *et al*., 2016). Theoretically, it may also ameliorate the symptoms of diseases associated with impaired synthesis of prosthetic groups for respiratory chain complexes. Complexes III and IV, as well as cytochrome *c*, contain heme. Defects in heme synthesis, iron and copper acquisition would lead to a functional deficiency of complexes III and/or IV.

It is difficult to predict which source of AOX enzyme would have the most benefits if used as a gene therapy tool in humans. AOX from *Trypanosoma brucei* (TAO) has been suggested to be the most appropriate to be used in human gene therapy (May *et al*., 2017). It functions at 37 °C within the human blood stream (Chaudhuri *et al*., 2006) and is the AOX protein that is the best structurally characterized (Shiba *et al*., 2013). It is also possible and desirable to engineer AOX proteins which can be specifically regulated for the purpose of gene therapy. This would potentially result in an AOX protein that could be turned 'on' and 'off' by cellular pyruvate levels or other effectors, and which could be beneficial under conditions of impaired ROS homeostasis (May *et al*., 2017).

Constitutive and unregulated functioning of AOX in the whole organism might be problematic, as it would decrease the yield of ATP. However, due to a very low affinity for its substrate, ubiquinol, AOX does not efficiently compete with complex III (Bahr & Bonner, 1973) under standard physiological conditions. For this reason, it is believed that AOX, even if present ubiquitously, functions only when the cytochrome pathway does not. However, if constitutively activated, AOX could be triggered by local metabolic conditions in specific cells, and participate significantly in respiration, it could alter ATP production and other processes dependent on respiratory energy, such as the buffering of calcium. Moreover, an increased metabolic rate with concomitant heat production could be detrimental in some tissues. Saari *et al.* (2017) reported that *Drosophila* males expressing AOX had apparent retention of sperm within the testis, which might be attributable to thermogenesis. An interference with cell signaling may also involve ROS. AOX has been reported to decrease mitochondrial ROS production even under the conditions of normal oxidative phosphorylation activity (Fernandez-Ayala *et al*., 2009; Sanz *et al*., 2010). This could affect signaling events in which ROS participate. This is why it may be important for gene therapy purposes to engineer an AOX which can be turned 'on' and 'off' as desired.

To date, AOX proteins from two different sources have been successfully expressed in human cells, namely from *C. intestinalis*, expressed in a HEK293T cell derivative (Hakkaart *et al*., 2006) and from the thermogenic plant *Arum concinnatum* in HeLa cells (Kakizaki & Ito, 2013). Thus far, the *C. intestinalis* AOX is the most studied in terms of potential gene therapy applications.

The immune response against transgene products is a critical issue for successful gene therapy. The bigger the phylogenetic distance between organisms, the greater the probability that the proteins of this organism would elicit an immune response. From this point of view, *C. intestinalis* AOX seems to be a better candidate for expression in humans than plant or trypanosomal AOX.

Although the AOX family contains a highly conserved active site there are remarkable differences in ubiquinol oxidase activity between different AOXs (May *et al*., 2017). More detailed kinetic and biophysical characterization of different AOX proteins is needed to understand how differences in structure and sequence are related to differences in function. Once the properties of AOX are well established, the sequence of the polypeptide can potentially be manipulated by design to be highly active, have low immunogenicity, and be susceptible to external regulation.

2.7 *Drosophila* genetic toolkit: The Gal4/UAS system for regulated transgene expression

The Gal4/UAS system (Brand & Perrimon, 1993) is a binary expression system primarily used in *Drosophila* which makes it possible to study effects of the overexpression or downregulation of any gene in a selected tissue and developmental stage (Brand & Perrimon, 1993). In the driver line, a construct containing a cell-specific promoter drives the expression of the gene encoding the yeast transcription factor Gal4 (Fig. 3 A). In the responder line, a transgene of interest is regulated by a promoter incorporating an upstream activation sequence (UAS). The Gal4 protein binds to the UAS sequence and drives expression of the transgene. Thus, the transgene should only be expressed in cells defined by the promoter regulating Gal4. Through their over-expression, misexpression or downregulation in a tissue- and time-specific manner, this allows different genes to be studied in an internally controlled manner. The two constructs are established in separate fly lines allowing for numerous combinatorial possibilities. The Gal4 protein has basal transcriptional activator activity at 18 °C, higher activity at 25 °C and even higher activity at 29 °C

(Busson & Pret, 2007). Temperature thus has a huge effect on the activity of Gal4 and on the expression of transgenes that it controls. It is therefore important to control temperature when performing experiments using this system. However, the Gal4/UAS system is far from perfect. There is variability in Gal4-mediated transcription between cells which cannot be explained (Brand *et al.*, 1994). Gal4 has dosage-dependent (Kramer & Staveley, 2003) biological effects, some of which are detrimental. Expression of high levels of Gal4 in *Drosophila* can modify the expression of many endogenous genes in a UAS-independent manner, among them components of important signaling pathways (Liu & Lehmann, 2008), even though there is no Gal4 ortholog nor UAS binding site in *Drosophila*. This potentially complicates the use of this system, since its original premise is that Gal4 is inactive in *Drosophila*. One of the mechanisms by which Gal4 might influence gene expression is via a selective block of protein transport into the cell nucleus (Uv *et al.*, 2000). Most promoters and enhancers that drive Gal4 expression are active many times throughout development. That is why a modified Gal4/UAS system, the GeneSwitch System (GS), that utilizes a modified Gal4 protein was developed (Osterwalder *et al.*, 2001). It requires antiprogestin, RU486, to activate it, in order to bind the DNA (Fig. 3 B). RU486 can be administered by either feeding or immersing the animals in a solution containing RU486. Reporter protein expression is dependent on the dose of the GeneSwitch activator drug RU486 (Osterwalder *et al.*, 2001). Again, the use of the GS system is justified under the assumption that GS is inert in the fly. However, GS is not completely inert in the absence of RU486, since it can still confer a significant decrease in target gene protein levels when RNA interference (RNAi) transgenes are used (Scialo *et al.*, 2016).

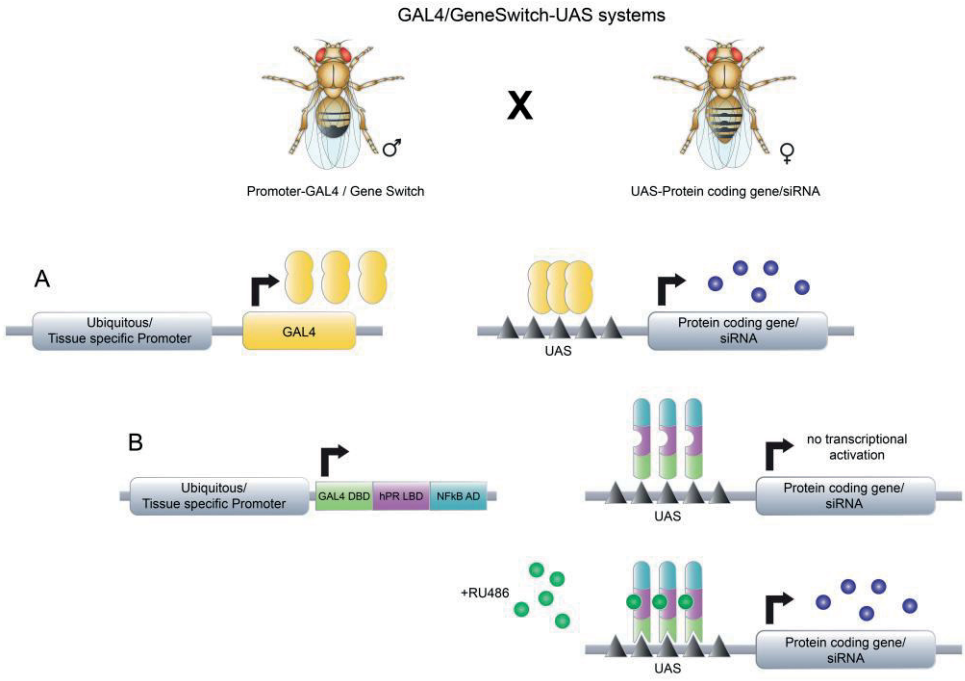


Figure 4. The Gal4/GeneSwitch-UAS expression systems in *Drosophila*. (A) GAL4 (yellow) binds at the UAS (dark grey triangles) which drives the tissue-specific expression/knockdown of the gene of interest. The GAL4-UAS system allows spatial control of gene expression. (B) GeneSwitch-UAS system allows temporal control of gene expression, in addition. In the absence of the activator RU486, the GeneSwitch (modified GAL4) protein is expressed in target tissues but is transcriptionally silent. Only when RU486 binds to the GeneSwitch protein, which is fused to the progesterone-binding domain, does it become transcriptionally active and thus able to drive the expression of UAS-linked genes. Modified from Nicholson *et al*., (2008) and Scialo *et al*., (2016).

3 AIMS OF THE STUDY

The aim of this thesis work was to test whether transgenic expression of AOX from the urochordate *Ciona intestinalis* in model organisms is able to influence cell migration and other developmental processes.

The more specific aims of this work were:

1. To engineer a catalytically inactive variant of the AOX protein (mutAOX) using site-directed mutagenesis, thus establishing a control for the entire study (I)

2. To create fly lines where AOX and mutAOX are inserted at the same integration site and thus serve as comparable controls for AOX activity in *Drosophila* using site-specific transgene insertion, (I, II)

3. To investigate the developmental effects of expressing the GeneSwitch transcription factor in *Drosophila*, and whether these are modified by concomitant AOX expression (II)

4. To test whether tissue-specific knockdown of JNK cascade genes produces cell migration defects during *Drosophila* development, and whether these can be corrected by concomitant AOX expression (III)

5. To test whether AOX expression influence mammalian cell migration, and if so under what conditions (III)

6. To determine the mechanism behind any such effect (III)

The starting point for the project was a preliminary observation made by my co-author, K. Kemppainen, regarding the interaction between AOX expression and GeneSwitch in *Drosophila*. This motivated the detailed study (II) undertaken in regard to Aims 3-4, as well as the need for an appropriate control (Aims 1-2, I). The

outcome of these studies then justified the more extensive investigation of cell migration and the JNK pathway (Aims 5-7, III).

4 MATERIALS AND METHODS

4.1 *Drosophila* stocks and maintenance (I-III)

Drosophila strains, e.g., drivers, balancers, transgenic recipient, RNAi and various control fly lines and their sources are described in original communications. Flies were maintained in standard high-sugar medium (Fernandez-Ayala *et al.*, 2009) on a 12-h light/12-h dark cycle at 25 °C, except where indicated in the figure legends. Where indicated, medium was supplemented with RU486 (Mifepristone, Sigma) at the concentrations indicated in figures and legends.

ΦC31 recombinase-mediated-site-directed transgenesis was used to generate transgenic fly lines (service provided by BestGene Inc, Chino Hills, CA), using recipient lines with the following integration sites: attP18 (chromosome X), attP40 (chromosome 2) and attP2 (chromosome 3), according to Pfeiffer *et al.* (2010) employing the wild-type and mutated AOX constructs cloned in pUASTattB and pUASTattB itself as empty-vector control. Following characterization, transgenic lines were maintained over balancers appropriate for chromosome X, 2 or 3, bearing standard markers (FM7, CyO, TM3Sb, respectively). Transgenic lines UAS-AOX^{F24} and UAS-AOX^{F6} were described previously (Fernandez-Ayala *et al.*, 2009) and referred to in the thesis and in publications as 'high' expression or 'old' wt AOX lines. Other lines created in the study are described under Results.

Crosses were implemented in triplicate, with flies being tipped into new vials on three successive days after mating. Nine vials per cross were analyzed in most experiments. Morphological abnormalities of the eclosed flies were scored as described in (II) and (III).

4.2 Cell culture

4.2.1 *Drosophila* S2 cells

Drosophila S2 cell line (Schneider, 1972), originally derived from late embryos, was cultured in Schneider's Medium (Sigma-Aldrich) at 25 °C. Cells were passaged every 3-4 days.

4.2.2 Mammalian cell lines

Human embryonic kidney 293 (HEK 293) cells originally derived by treating a human embryonic kidney homogenate with sheared adenovirus DNA (Graham *et al.*, 1997). This cell line has a highly aberrant karyotype and constitutively expresses Ad5 E1A/E1B proteins, which deregulate pRB/p53pathways (Stepanenko & Dmitrenko, 2015). HEK293T (293T, ATCC® CRL-3216™) cells used for the work in I, are the derivative of HEK 293 cells with the difference that they express the SV40 T-antigen.

AOX-positive and -negative mouse embryonic fibroblasts (MEFs), sourced either from embryos transgenic for *C. intestinalis* AOX, inserted at the *Rosa26* locus (Szibor *et al.*, 2017), or from their nontransgenic littermates, were studied at passage 6. MEFs, immortalized by retroviral transduction with HPV16 oncoproteins E6 and E7 (iMEFs) were supplied by P.K. Dhandapani, University of Tampere (III).

The AP-1 reporter HEK293 recombinant cell line (JNK signaling pathway; BPS Bioscience), here abbreviated HEK-AP1, contains a firefly luciferase gene under the control of AP-1-responsive elements that are stably integrated into HEK293 cells (III).

The BJ-5ta human fibroblasts (ATCC CRL-4001) has been immortalized with human telomerase reverse transcriptase (hTERT) (III).

Mammalian cell lines were maintained at 37 °C in 5% CO_2 and cultured as detailed in the original publications.

4.3 Molecular cloning

The *C. intestinalis* AOX cDNA (Hakkaart *et al.*, 2006) was recloned into suitable expression vectors for *Drosophila* (S2 cells and whole flies) and mammalian cells. For details see (I). *In vitro* mutagenesis of these constructs employed a PCR-based, site-directed mutagenesis approach with customized oligonucleotides using Quick Change Site-Directed Mutagenesis Kit (Agilent Technologies) (I). All constructs were verified by sequencing prior to use.

4.4 Gene expression assays

4.4.1 RNA expression analysis by Quantitative Reverse Transcription-PCR (qRT-PCR)

Extraction of RNA from 2-day old adult flies and S2 cells and measurements of transcript levels relative to an internal standard (RpL32 mRNA) were performed using standard methods (Fernandez-Ayala *et al.*, 2009), as described in (I) and (III).

4.4.2 Protein analysis by Western blotting

To measure transgene expression at the protein level in the adult fly, total protein was extracted from 30 females or 40 males per sample (I, Fig. 2 D and E). Frozen fly samples were used for all experiments except for detecting mutant AOX in females, for which fresh samples were used (I, Fig. 2 D).

For detecting phosphorylation at c-Jun, 300,000 MEFs or HEK-AP1 cells or 250,000 BJ-5ta cells were plated on 6-well plates (CellStar; Greiner Bio-One, III, Fig. 5). After 24 h, the medium was replaced with medium containing either 0.2% DMSO, 20 μM SP600125 in 0.2% DMSO, 20 μM JNK inhibitor V, 8 nM PMA, or no added drug and the plate was incubated for 2 h (or 40 min, in the case of PMA). Cells were carefully rinsed in ice-cold PBS and then scraped on ice using a CytOne cell scraper in resuspension buffer.

Primary antibody incubations were carried out overnight at 4 °C. Membranes were then washed 3 x 10 min in PBS-Tween and incubated with HRP-conjugated secondary antibody for 1 h at room temperature in PBS-Tween with 5% nonfat (w/v) milk. After this, the unbound secondary antibody was removed by three successive 10 min washes with PBS-Tween. Protein was detected and visualized using Immun-Star™ HRP Chemiluminescent Kit (Bio-Rad). The chemiluminescence of all blots was documented both with film and by using a Bio-Rad ChemiDoc imager.

Primary antibodies used were: customized rabbit anti-AOX (21st Century Biochemicals, 1:10,000, Dassa *et al.*, 2009), rabbit anti-α-actinin C-20-R used as loading control (Santa Cruz Biotechnology, 1:5,000 (I) 1:7,000 (III), mouse anti-ATP5α used as loading control (Abcam, 1:50,000(I)), phospho-c-Jun (Ser 73) rabbit monoclonal antibody (catalog number 3270; Cell Signaling Technology 1:1,000, III), and phospho-c-Jun (Ser 63) rabbit polyclonal antibody (catalog number 9261; Cell Signaling Technology, III). HRP-conjugated secondary antibodies were: peroxidase-labeled Goat Anti-rabbit IgG (catalog number PI-1000) and Horse Anti-mouse IgG (catalog number PI-2000 (both from Vector Laboratories, 1:10,000 (I, III)).

4.5 Transfection/transduction

Transgenes were introduced into *Drosophila* and mammalian cell-lines, for various purposes, by plasmid DNA lipofection or by lentivector transduction, as detailed in (I) and (III). Briefly, to assess the expression and activity of AOX and a mutated variant (see Results and I, Fig. 2 A), plasmid DNA was transiently transfected into HEK293T cells using 60 µl Lipofectamine® 2000 (Invitrogen) under manufacturer's recommended conditions (I). To assay the effects of transgene expression on AP-1 directed transcription using a luciferase reporter approach, test plasmids and an appropriate cocktail of reporter-system plasmids (Chatterjee & Bohmann, 2012) were transiently transfected into *Drosophila* S2 cells (III, Fig. 7 A-D), using the FuGENE® HD transfection reagent (Promega) according to the manufacturer's instructions. To conduct similar assays in mammalian cells, as well as for Western blotting to study c-Jun phosphorylation, transgenes were stably introduced into the HEK AP-1 reporter cell-line or into BJ-5ta cells by pWPI-based lentiviral transduction, followed by cell sorting and cloning at limiting dilution (III).

4.6 Migration assays

4.6.1 Wound-healing assay

For the wound-healing assay, samples of 90,000 cells were plated on 24-well plates (CellStar; Greiner Bio-One) as technical triplicates (3 wells per sample). After 24-48 h, cells were treated with various drugs as detailed in (III), after which a linear scratch was made in the center of each well with a p10 (1 to 10 μl) pipette tip. The cells were then washed three times with medium to remove detached cells, and fresh medium containing the appropriate reagent was added to each well.

4.6.2 Single-cell migration assay

To measure single-cell migration, samples of 15,000 primary or immortalized MEFs (iMEFs) were plated on 6-well plates (CellStar; Greiner Bio-One) as technical triplicates (3 wells per sample). To minimize plate-specific effects, each plate contained all the genotypes used in a single experiment. Cells were imaged as described below, under section 4.7.

4.7 Microscopy

Light microscopy images of eclosed adult flies and their appendages were taken with a Nikon Digital DS-Fi1 High-Definition Color Camera, using the Nikon stereoscopic zoom microscope SMZ 745T run by NIS-Elements D 4.20 software. For live imaging of GFP fluorescence (III), anaesthetized adult flies or cleaned larvae were analyzed using a Zeiss Axio Imager2 microscope (50x magnification, producing Z projection images). Embryos collected from mating chambers or gently detached pupae were imaged in 35-mm glass-bottom microwell dishes filled with Halocarbon

Oil 700 (Sigma-Aldrich), using an Andor spinning disc confocal microscope at 20x magnification.

Wound closure time-lapse images were taken with a ChipMan Technologies Cell-IQ controlled-environment observation incubator equipped with a Retiga EXi 1392 charge-coupled-device camera, using a Nikon CFI Plan Fluorescence DL objective at 10x magnification until the wound closed (approximately 48 h) or for 24 h when metabolic inhibitor drugs were applied. Images were taken every 30 min. For further details, see (III). To generate images for measuring single-cell migration, cells were transferred 24 h after plating to a Nikon BioStation CT instrument equipped with a Nikon DS-1QM camera and imaged at 4x magnification every 6 min. Movement of living cells was analyzed using CellTracker image-processing software with semi-automated migration detection.

4.8 Luciferase assays

4.8.1 Luciferase reporter assays in S2 cells

The activities of cell-based reporters were measured by Dual Glo Luciferase Assay System kit (Promega). Transfected S2 cells (either untreated or treated with hep dsRNA to downregulate JNK pathway or pACT-Gal4 together with pUAST-Hep^{act} for activated transcription) were transferred to wells of 96-well luciferase assay microplates (Lab Systems) and the luciferase assay was carried out in triplicate according to the manufacturer's protocol. Luminescence was measured using a Thermo Labsystems Luminoskan Ascent plate reader (III, Fig. 7 A-D).

4.8.2 Luciferase reporter assays in mammalian cells

Firefly luciferase reporter assays were carried out in HEK-AP1 cells and in AOX/mutAOX-expressing clones derived from them using the Dual-Glo luciferase assay system (Promega), according to the manufacturer's protocol. Luminescence was measured using a PerkinElmer UV/visible plate reader (III, Fig. 7 E).

4.9 Respirometry

To test whether the mutAOX protein has enzymatic activity, oxygen consumption was measured on transiently transfected permeabilized HEK293T cells 48 h after transfection, in a Clark-type electrode (Hansatech Oxytherm system). Complex II-driven respiration was measured in the presence of ADP and succinate. AOX-driven (antimycin-resistant) respiration was measured after the further addition of antimycin A, with subtraction of any residual oxygen consumption after adding AOX inhibitor, n-propyl gallate (for details, see I). Similarly, respirometry on S2 cells and homogenates from 1-4 day-old *Drosophila* males was conducted using the same system (I).

4.10 Experimental methods conducted by co-authors

In the published papers, molecular modelling (I, Fig. 1), Cox7a deficiency survival assay (I, Fig. 5 A), fly locomotor activity assay (I, Fig. 5 B), generation and respirometry of primary and iMEFs (III, Fig. 8 A and B), and wound-healing assays conducted in the presence of respiratory inhibitors (III, Fig. 8 C) were conducted by my co-authors.

5 RESULTS

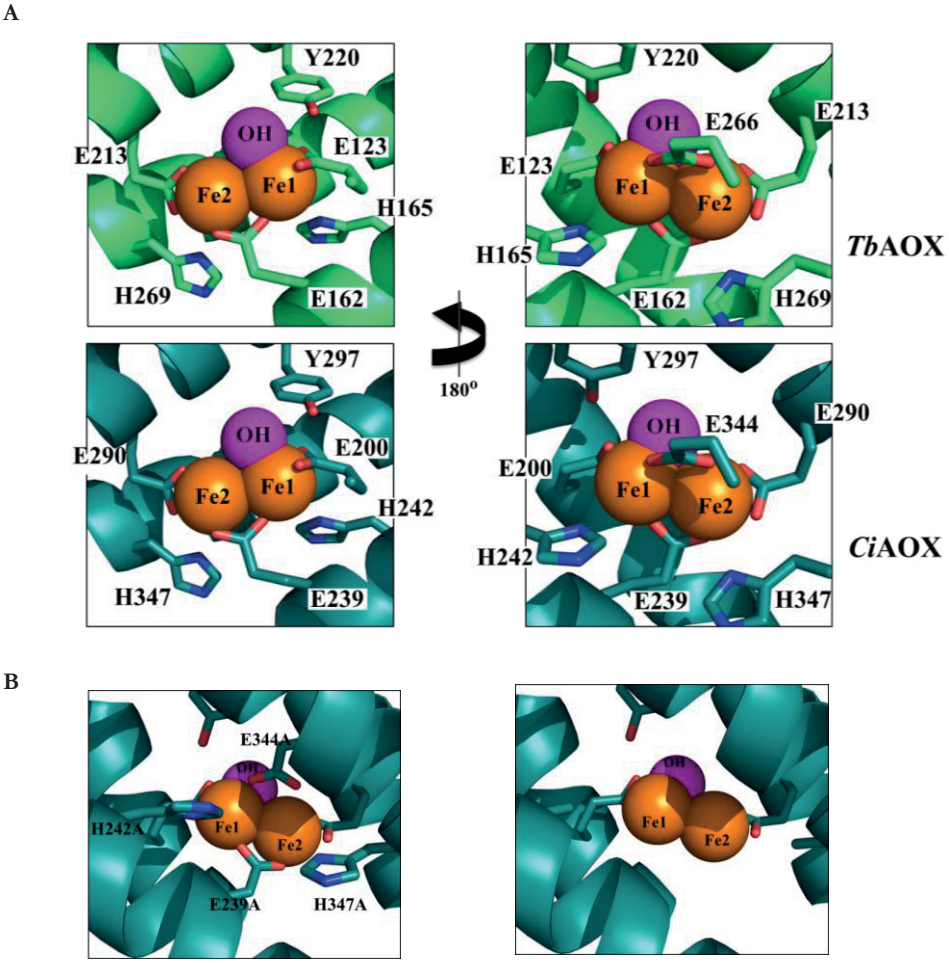
5.1 Structural modelling and mutagenesis of active site of *Ciona intestinalis* AOX (I)

AOX is a diiron nonheme carboxylate protein which catalyzes four-electron oxidation of reduced ubiquinone (ubiquinol) reducing oxygen to water at the same time (Kern *et al.*, 2007).

Introduction of AOX from *C. intestinalis* (Tunicata: Ascidiacea) by transgenesis compensates many of the phenotypes resulting from complex III or IV deficiency in the fruitfly (Fernandez-Ayala *et al.*, 2009; Kemppainen *et al.*, 2014), cultured mammalian cells (Haakkart *et al.*, 2006 Dassa *et al.*, 2009) and in the mouse (El-Khoury *et al.*, 2013; Szibor *et al.*, 2017). In order to understand whether the ability of AOX to rescue COX deficiency (Kemppainen *et al.*, 2014), as well as cleft thorax and other developmental defects (see below; II, III), depends on its known enzymatic activity, we engineered a mutated variant of AOX (mutAOX) predicted to be disabled for enzymatic activity. We used findings from two previous studies to help us design mutAOX. In the first, AOX was shown to be phylogenetically well conserved and its active site residues have been characterized in a number of species (McDonald *et al.*, 2009). In the second, the crystal structure of a representative AOX from *Trypanosoma brucei*, the parasite that causes African sleeping sickness, was resolved (Shiba *et al.*, 2012).

From these starting points we aligned the predicted *Ciona* AOX amino-acid sequence with *T. brucei* AOX, which revealed conservation of residues (I, Fig. S1 of the supplementary data). To engineer a fully catalytically inactive protein, we mutated four amino acids to alanine E239A, H242A, E344A, H347A (Fig. 5 B) (for details see I, Materials & Methods). Sequence alignment reveals that these four residues are fully conserved across the sequenced family of AOX from different species (McDonald *et al.*, 2009). We substituted these amino acids with alanine, because alanine is a small and inert amino acid, a standard procedure in the field of mutagenesis. The mutations were predicted to disable AOX activity. The glutamate and histidine residues we mutated are directly involved in binding diiron moiety (Fig. 5 A). We hypothesized that changing these residues would destabilize iron binding

and abolish enzymatic activity, leaving the protein still able to go to the mitochondria and fold correctly but unable to exhibit its normal enzymatic activity. We then tested to see if this was indeed the case.



Selected residues in *Ci*AOX for alanine substitution mutagenesis

Modeled alanine substitutions in *Ci*AOX structural model

Figure 5. Structural modelling and mutagenesis of active site of *Ciona intestinalis* AOX (*Ci*AOX). (A) Conserved residues binding the diiron centre show an identical disposition in the *Ciona* model (*Ci*AOX, green) as in the *Trypanosoma brucei* AOX (*Tb*AOX, blue) (B) The residues selected for alanine-substitution mutagenesis in the *Ci*AOX (here shown in blue), the resulting modelled structure (right) (Modified from I, Fig. 1).

5.2 MutAOX is stably expressed in mammalian cells and flies (I)

To study the properties of mutAOX when expressed, I first verified the expression of the mutAOX construct by transient transfection into cultured HEK293T cells. Based on Western blotting (Fig. 6 A), mutAOX protein was the same size and expressed at comparable levels to wild-type (wt) AOX. Next, the mutAOX transgene, under the control of the GAL4-dependent UAS promoter, was introduced into the *Drosophila* genome by targeted insertion (for details see I, Materials & Methods). Control lines were created, containing wt AOX (described as 'new' wt AOX line in I) and empty vector, inserted at the same site. Insertions were validated by PCR and sequencing. I then measured transgene expression directed by the ubiquitous *da*GAL4 driver, at both RNA and protein levels, using qRT-PCR (Fig. 6 B, C) and Western blotting (Fig. 6 D, E). In both females (Fig. 6 B) and males (Fig. 6 C), the expression of wt and mutAOX were similar at the RNA level, but 3-4 fold lower than AOX in the previously created transgenic lines, engineered by random P-element insertion ('old' wild-type AOX lines, I). At the protein level, mutAOX has slightly lower expression than wild-type AOX in both sexes, and expression was again less than in the previously created 'old' wt lines. (Fig. 6 D, E).

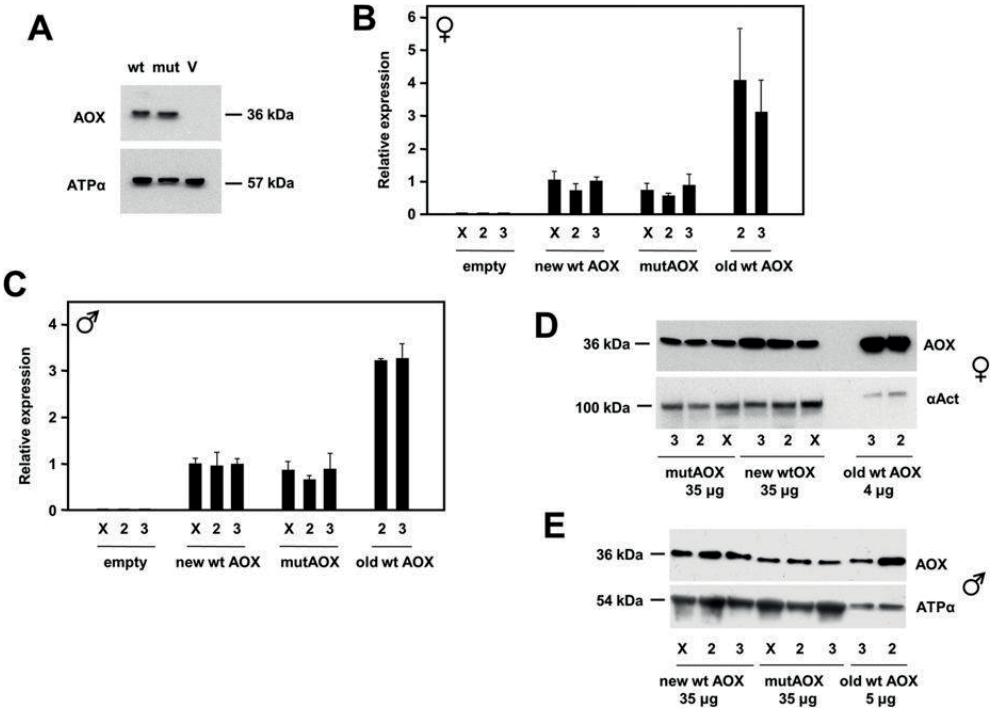


Figure 6. Expression of wt and mutAOX transgenes in mammalian cells and *Drosophila*. (A) Western blot of protein lysates of HEK293T cells transfected with wt, mutAOX or empty vector constructs (wt, mut, V), probed for AOX and for ATP synthase subunit α (ATP α) as loading control. (B, C) Relative RNA level of AOX expression, based on qRT-PCR (means ± SD), in (B) females and (C) males of *Drosophila* lines transgenic for wtAOX, mutAOX, or empty vector. New wt and mutAOX lines were created by site-specific integration at defined chromosomal sites using the ΦC31 system; old wt AOX lines were UAS-AOX^{F6} (chromosome 2) and UAS-AOX^{F24} (chromosome 3). For males, all values were significantly different from empty-vector lines; old wt AOX lines were significantly different from new wt AOX lines ($p < 0.001$, ANOVA followed by *post-hoc* Bonferoni-corrected *t* test). mutAOX and new wt AOX lines were not significantly different from each other. Statistical analysis for females gave similar results, although greater sample-to-sample variation for old wt AOX lines yielded only $p < 0.05$ comparing them with new wt or mutAOX lines. (D, E) Western blot of protein extracts from the same flies, probed for AOX or, as loading control, either ATP α or α-actinin (α Act). Unmodified from I, Fig. 2.

MutAOX was stably expressed in cultured human cells and in transgenic flies, and it lacked enzymatic activity as expected (I, Fig. 4; enzymatic activity experiment conducted jointly with E. Dufour). Furthermore, mutAOX could not compensate pupal lethality caused by knockdown of cytochrome *c* oxidase subunit COX7a (I, Fig. 5 A), nor locomotor dysfunction resulting from neuronal specific knockdown

of COX7a (I, Fig. 5 B; experiment conducted by C. Yalgin), confirming that the observed rescue depends on the enzymatic activity of AOX.

5.3 Expression of AOX rescues cleft thorax caused by *tub*GS/RU486 (II)

The GeneSwitch (GS) system allows temporal control of gene expression thanks to a modified GAL4 protein that is active only when the synthetic progesterone analogue, RU486, binds to the fused progesterone steroid receptor. GeneSwitch adds temporal control to the GAL4/UAS system (Yamada *et al.*, 2017). Transgene expression was induced during development by the GS, under the control of a ubiquitously active α-*tubulin* promoter (*tub*GS), by feeding RU486 to mothers while laying eggs, and to larvae until pupariation. For experiments involving RU486 we used a diet rich in nutrients (Fernandez-Ayala *et al.*, 2009) because RU486 is not detrimental to fly lifespan on high nutrient food (Yamada *et al.*, 2017).

In the preliminary experiment it was observed that *tub*GS expression in the presence of RU486 causes cleft thorax which is rescued by AOX (Fig. 7 A). In a follow up study, I further observed, using the same system with induction by 10 μM RU486, extensive pupal lethality and a spectrum of additional malformations such as an enlarged tibia and femur, both fused together preventing movement of the fly in the majority of cases, bristle malformations, cleft abdomen, notches on anterior and posterior wing margins and externalized trachea (Fig. 8). These highly dysmorphic flies were alive nonetheless. For all the experiments throughout this thesis work, only vials with embryo numbers from 50-100 were analyzed to eliminate a crowded population which could affect resource availability during feeding, thus affecting the results.

Coexpression of AOX rescued the phenotype substantially, with over 50% of the eclosing progeny now showing no cleft, and less than 20% having severe cleft. Ndi1 targeted to mitochondria or GFP expressed in the cytoplasm did not rescue the phenotype. Nor did a single copy of AOX, when constitutively expressed under the α-*tubulin* promoter at a much lower level than when driven by *tub*GS. However, five doses of the *tub*AOX transgene gave rescue (II, Fig. 4 C) comparable with the UAS-AOX lines (which express AOX at a higher level than *tub*AOX and UAS-AOX$^{7.1}$ fly lines). To test if rescue is due to catalytic activity of AOX we tested mutAOX in the assay. The enzymatically inactivated variant of AOX did not rescue any of the

phenotypes implying that rescue was due to enzymatic activity of the AOX and that AOX operates in a dose dependent manner (Fig. 7 B).

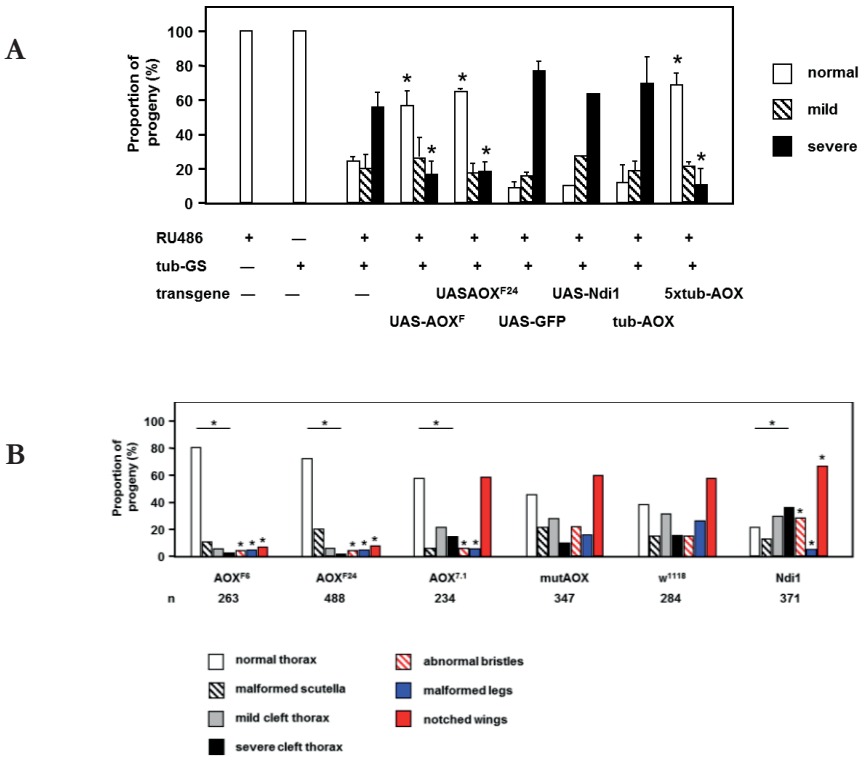


Figure 7. (A) AOX partially rescues cleft thorax and developmental lethality caused by activated *tub*GS. Proportion of adult progeny exhibiting the indicated phenotypes, with hemizygous transgenes as indicated, cultured with or without 10 µM RU486. $n \geq 3$ replicate vials for each studied genotype (except UAS-Ndi1, $n = 2$, hence no error bars shown). *tub*AOX transgenic lines had either a single hemizygous copy of AOX (*tub*-AOX) or five copies (5x*tub*-AOX). Asterisks indicates data classes significantly different from the equivalent class for control lacking any transgene additional to *tub*GS (Student's *t*-test with Bonferroni correction, $p<0.01$). (B) AOX alleviates developmental abnormalities induced by activated *tub*GS. Proportion of progeny hemizygous for both *tub*GS and the indicated transgenes, which exhibited the indicated developmental abnormalities at 10 µM RU486. The total numbers of flies of each genotype analyzed, in a single large-scale experiment (n). Asterisks indicate significant differences (p <0.001) from the w^{1118} strain hemizygous for *tub*GS, based on chi-squared analysis for each phenotypic category or for the four thoracic phenotypes (normal thorax, malformed scutellum, mild cleft, and severe cleft) considered as a whole. Modified from II, Fig. 4 and 5.

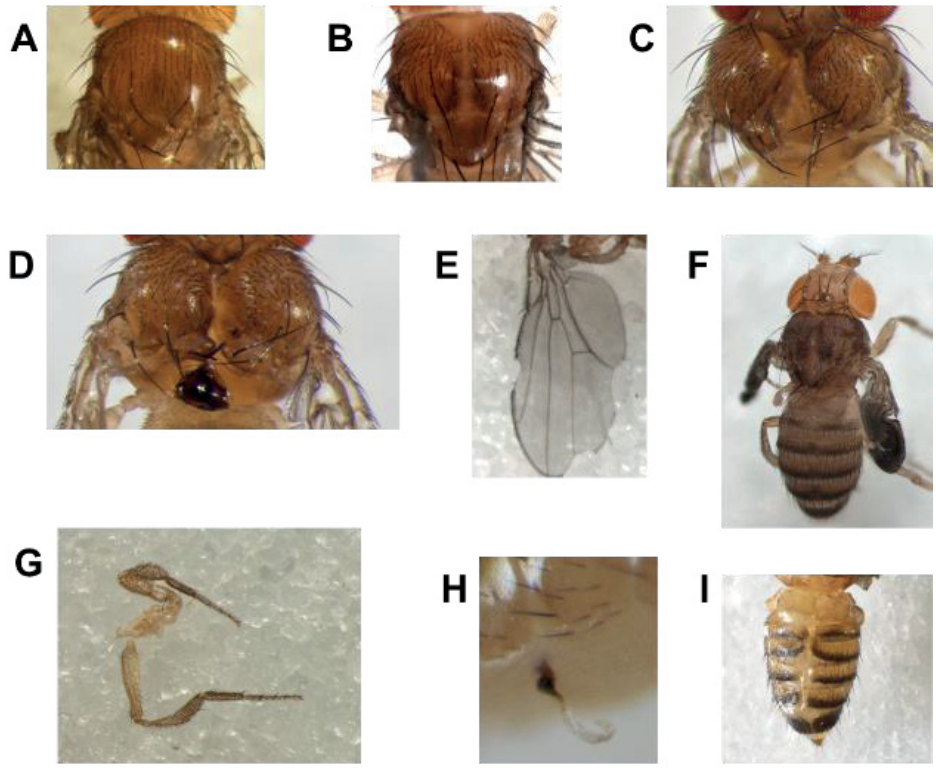


Figure 8. Examples of malformations caused by the *tub*GS driver in the presence of 10 µM RU486. (A-D) Thoracic abnormalities: (A) missing scutellar part, (B) mild cleft, (C) severe cleft, and (D) necrotic tissue, always localized at the scutellum or notum. (E, F) Wing abnormalities: (E) notched wings, with notches localized on the marginal anterior or posterior side or both, (F) noninflated wings. (G) Leg abnormalities, including overgrown and fused leg segments. (H) Externalized tracheal appendice, always in the ventral abdomen proximal to the posterior leg. (I) Abdominal cleft: midline splits between all dorsal tergite plates; laterotergites do not fuse at the dorsal midline and remain as hemitergites. *tub*GS; a-*tubulin*-GeneSwitch. Unmodified from II, Fig. 5.

5.4 AOX expression alleviates cleft thorax due to downregulation of JNK signaling (III)

The findings presented in the foregoing section showed that a high dosage of *C. intestinalis* AOX expressed in *Drosophila* could alleviate developmental abnormalities caused by an engineered transcription factor from yeast. This prompted us to test if AOX could correct a well described *Drosophila* model of cleft thorax phenotype. Downregulation of JNK pathway genes, or misregulation of the transcription factors

encoded by *pnr* or *usp*, consistently leads to cleft thorax and was thus an obvious target. We first downregulated selected genes of the JNK signaling cascade in the mediodorsal region during *Drosophila* development using *pnr*^{MD237} GAL4 driver. This downregulation resulted in a cleft thorax phenotype as published previously. Using the GFP reporter already present in the *pnr*^{MD237} GAL4 fly line we verified the expression pattern of it (III, Fig. 1 B; see Table S1 in the supplemental material). We used *pnr*^{MD237} GAL4 to downregulate *PDGF and VEGF receptor related* (*pvr*; a receptor tyrosine kinase), *misshapen* (*msn*; JNKKKK) *hep*, *bsk*, *Jun-related antigen* (*Jra*; *Drosophila fos*) and *kayak* (*kay*; *Drosophila jun*) and to overexpress *puckered* (*puc*) (III, Table S2). These all produced a cleft thorax phenotype of various severities (Fig. 9) which was dependent on the tested construct/insertion and temperature.

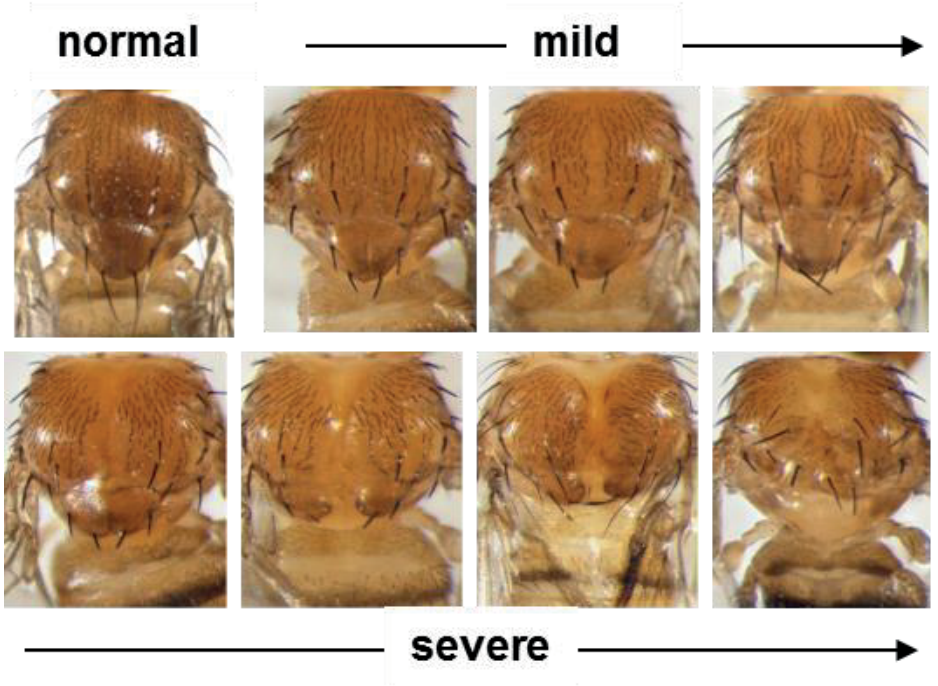


Figure 9. Examples of thoracic phenotypes throughout the study scored as normal, mild, or severe. Arrows indicate the trend within each class toward more severe cleft thorax phenotypes. Modified from III, Fig. 1 C.

To attempt the rescue we combined these with expression constructs for AOX or for a control transgene, GFP. The mutAOX fly line (see above) was not available at the time of this study.

For *bsk* and *hep*, I tested multiple RNAi lines (III, Table S1), each producing cleft thorax when combined with the *pnr*^{MD237} GAL4 driver. Expression of AOX (III Fig. 2 A and Fig. 10 A, B) but not that of GFP (III, Fig. 2 B) alleviated the phenotype substantially. Using two different RNAi lines for each gene gave the same effect of AOX (Fig. 10 E-G). To exclude the promoter dilution effect we measured the amount of AOX RNA driven by *pnr*^{MD237} GAL4 in pupae with and without one of the double-stranded RNA (dsRNA) constructs for *hep*, which showed no significant difference (III, Fig. 2 D). Very few flies from the *msn* knockdown eclosed, while AOX or GFP expression gave no significant change in phenotype, despite a trend toward the wild-type for AOX (Fig. 10 C) and increased severity in the case of GFP (Fig. 10 F). A *pvr* knockdown was pupal semilethal when combined with the *pnr*^{MD237} GAL4 driver, even when milder knockdown of the *pvr* was induced by growing the flies at 18 °C. Nevertheless, the number of eclosing progeny was not sufficient to enable a statistically meaningful analysis of the thoracic phenotype according to severity, but the mean proportion of progeny with cleft thorax was about 80% in this and parallel *pvr* knockdown experiments. Co-expression of AOX, but not GFP, significantly rescued semilethality (III, Fig. 2 C). AOX was not able to rescue *kay* and *jra* knockdown nor the lethality caused by overexpression of the AP-1 target Puc, which antagonizes Bsk by feedback mechanism (Martín-Blanco *et al.*, 1998).

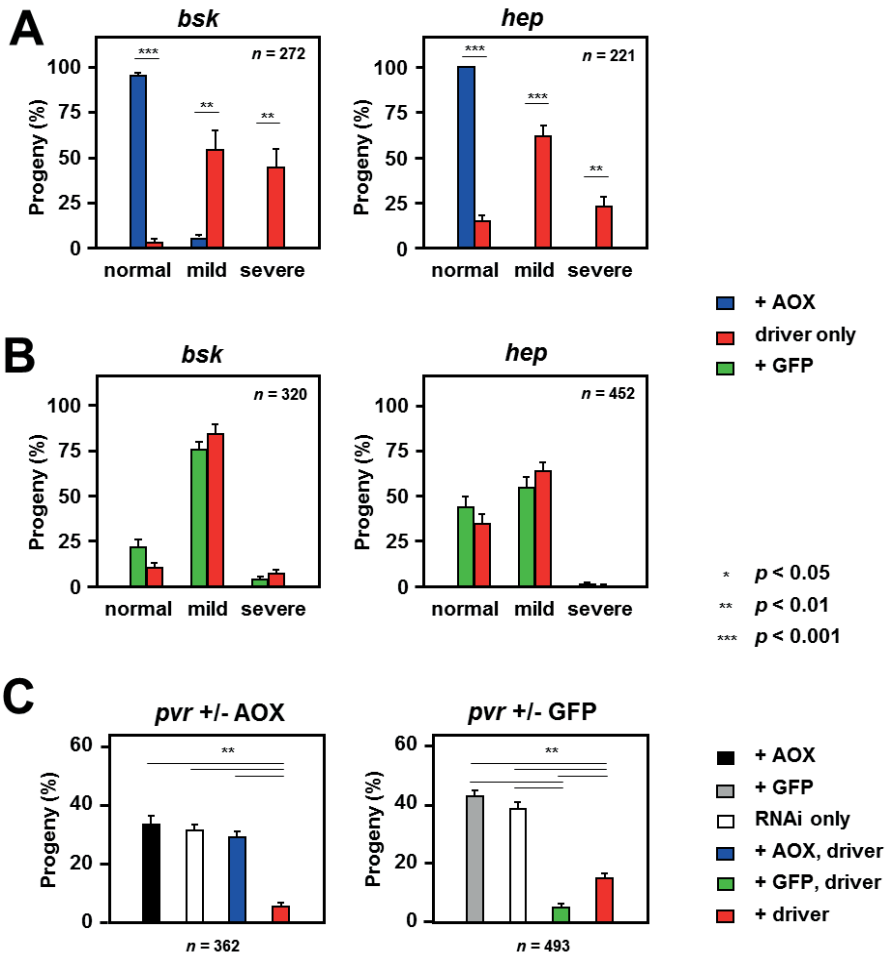


Figure 10. AOX rescues cleft thorax induced by JNK signaling downregulation. Effects of co-expressing (A) AOX or (B) GFP on the proportion of different phenotypic classes resulting from knockdown of *bsk* and *hep*, using the *pnr*^{MD237} GAL4 driver and RNAi lines KK 104569 (*bsk*) and GD 47507 (*hep*). The data represent the means ± SEM for nine replicate vials in each experiment. n, the total number of flies analyzed. Statistically significant differences between the proportions of AOX or GFP expressing and nonexpressing flies of different phenotypic classes are shown. P values, as indicated, determined by Student's *t*- test, two-tailed, paired, with the Bonferroni correction. (C) Effect of co-expressing AOX or GFP on pupal semilethality caused by knockdown of *pvr* (RNAi line KK 105353) using the *pnr*^{MD237} GAL4 driver. The data represent the means ± SEM for nine replicate vials in each experiment. n, the total number of flies analyzed in each case. Statistically significant differences between classes are indicated, with P values determined by ANOVA with the Tukey post hoc honestly significant difference test.

5.5 Is AP-1 the target of AOX? (III)

5.5.1 AOX does not rescue cleft thorax caused by AP-1 transcriptional factor or manipulation of its downstream target Puc

We next downregulated c-Jun or its dimerization partner, c-Fos, encoded in *Drosophila* by *Jra* and *kay*, respectively using the same *pnr*^{MD237} GAL4 driver. Co-expression of AOX had only a slight effect on the severity of cleft thorax induced by knockdown of *kay* or *Jra* using the *pnr*^{MD237} GAL4 driver (Fig. 11 A and B). AOX was also unable to rescue the lethality caused by overexpression of the AP-1 target, Puc, which antagonizes the action of Bsk (Fig. 11 C). This result leaves open a possible direct involvement of AOX on AP-1 transcriptional factor.

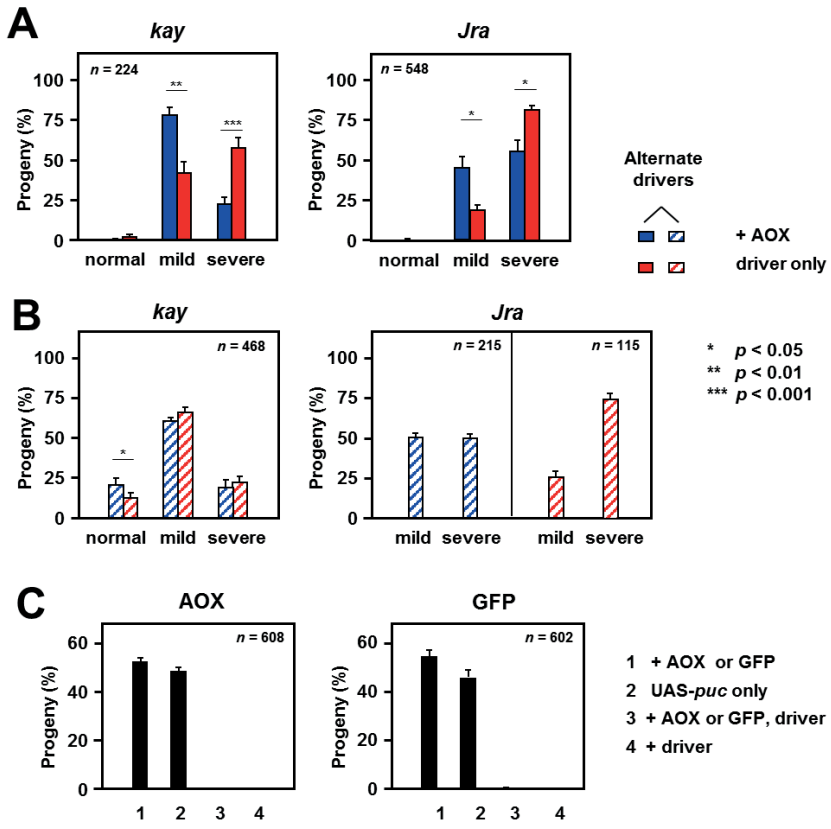


Figure 11. AOX does not rescue cleft thorax induced by altered expression of AP-1 or its target puc. (A, B) Effects of co-expressing AOX on phenotypic classes resulting from knockdown of *kay* (at 25 °C) and *Jra* (at 18 °C), using the *pnr*^{MD237} GAL4 driver and RNAi lines GD 6212 (*kay*) and KK 107997 (*Jra*) (A) and alternate RNAi lines GD 19512 (*kay*) and GD 10835 (*Jra*) (B). GD 10835 (*Jra*) construct is carried on chromosome X, two parallel crosses were required to test the effects of AOX expression in each sex, and statistical analysis was not meaningful in this case. The data represent the means ± SEM for 9 replicate vials in each experiment. n, total number of analyzed flies. Statistically significant differences between the proportions of AOX expressing and nonexpressing flies of different phenotypic classes are shown. P values determined by Student's *t*-test, two-tailed, paired, with the Bonferroni correction. *Jra* knockdown using RNAi line KK 107997 was lethal at 25 °C and AOX did not rescue this lethality. (C) Effect of coexpressing AOX or GFP on pupal lethality caused by overexpression of puc driven by *pnr*^{MD237} GAL4 driver. All indicated progeny classes contained the overexpression construct, UAS-puc. The data represent the mean ±SEM for nine replicate vials in each experiment. n, the total number of analyzed flies.

5.5.2 AOX does not influence AP-1-dependent transcription in cultured cells

In attempt to reveal the mechanism of the AOX-mediated rescue of cleft thorax, I tested whether AOX expression can change the transcriptional activity of AP-1 in the *Drosophila* and mammalian system. I set up luciferase reporter-based assays for both S2 and HEK293T cells. I tested whether AOX expression in *Drosophila* S2 cells was able to alter AP-1-dependent transcription under different conditions of JNK pathway activation, using the reporter system developed by Chatterjee & Bohmann (2012). AOX did not influence AP-1 transcription in the conditions of basal and activated JNK pathway. JNK pathway activation was achieved using the pUAST-Hep^{act} plasmid in combination with pAct-Gal4, which promotes pUAST-Hep^{act} transcription of pUAST-Hep^{act} by constitutive expression of Gal4. (Fig. 12 A-D).

To conduct a similar experiment in mammalian cells, I made use of an AP-1 Reporter HEK293 cell-line derivative (see III, Materials & Methods), here designated HEK AP-1. The cell line contains a stably integrated firefly luciferase gene under the control of AP-1-responsive elements. It was not possible to obtain consistent results with transiently transfected cells (data not presented). I therefore created stable AP-1 reporter cell lines, expressing AOX and mutAOX, using lentiviral constructs in which the transgene is co-expressed with GFP. Cells were cloned at limiting dilution. Different clones had highly variable degrees of AP-1 transcriptional activity, most likely reflecting the diversity of copy number and integration sites, as well as possible karyotypic instability. This is a limitation of the system.

However, I was able to replicate the findings from S2 cells in the mammalian model: AOX expressing clones showed no significant difference in AP-1 transcriptional activity compared with mutAOX expressing clones or parental cells (Fig. 12 E).

Using this model, I also tested the effects of inhibiting JNK pharmacologically with two inhibitors, SP600125 and AS601245 (JNK inhibitor V or inh V) (III). Whilst inh V decreased AP-1 transcription, SP600125 increased it, except when cells were pre-stimulated with PMA (Fig. 12 E), but again no differential effect was seen between AOX expressing, mutAOX expressing and control cells.

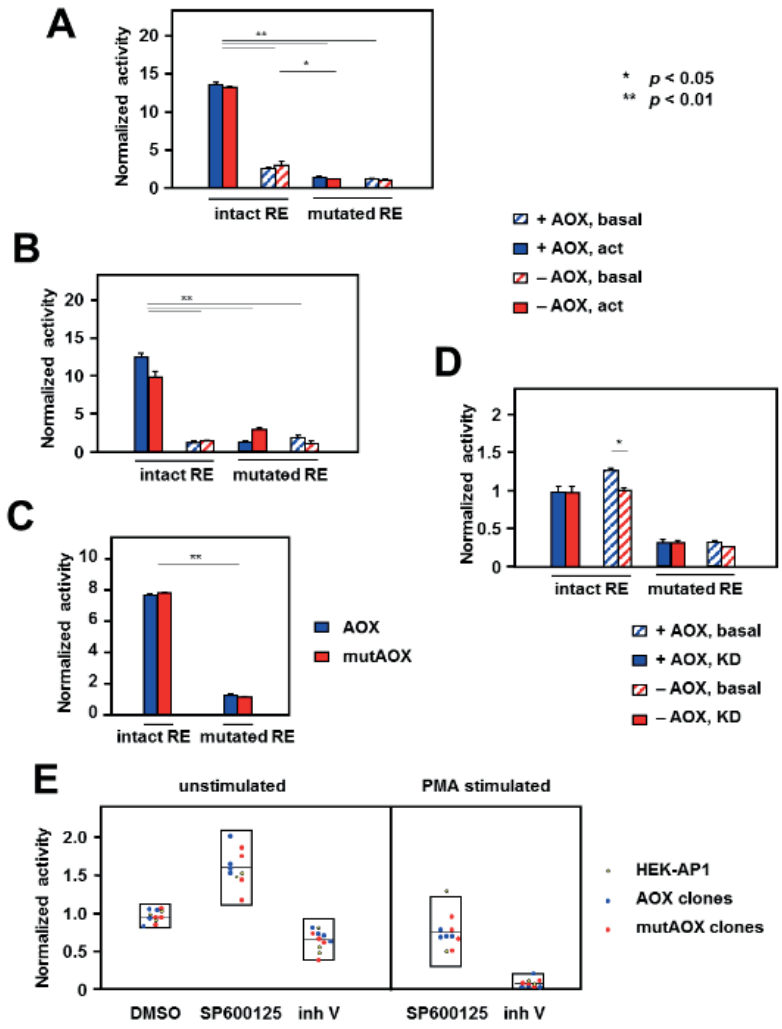


Figure 12. AOX effect on AP-1-dependent transcription. (A-D) Firefly luciferase activity in S2 cell extracts co-transfected with (A) pMT-AOX (or empty vector) (B) pAC/AOX (or empty vector) (C) pUASTattBAOX (or pUASTattB-mutAOX), (A-C) and with pUAST-Hep^{act} ± pAct-Gal4 to activate it or not (D) or following 5 days of dsRNA treatment (knockdown) for *hep* and with reporter constructs containing intact (TRE-fluc) or mutated (mRE-fluc), phorbol ester-response elements (RE), later serving as a background control and pAct-RL for normalization. All data were first normalized for transfection efficiency, using a cotransfected *Renilla* luciferase activity, and then renormalized against basal activity for the relevant controls: (A, B, D) the empty vector or (C) pUASTattB-mutAOX. AOX expressing and AOX nonexpressing cells were not significantly different from each other apart from one case in panel D (Student's *t*-test, two-tailed, unpaired). Statistically significant differences between data classes were determined by one-way ANOVA with the Tukey post hoc honestly significant difference test. act, activated. (E) Firefly luciferase

activity in extracts of reporter cells and HEK AP-1 derived clones transduced with lentiviral constructs expressing AOX or mutAOX. Treatments: 0.2% DMSO, 20 μM SP600125 or 20 μM inh V. Data normalized against the values for the corresponding non-treated cells (unstimulated) or for the corresponding cells treated with 8 nM PMA (stimulated). Box plots indicate the means and the 95% confidence intervals for each data set. Modified from III, Fig. 7.

5.5.3 AOX expression does not affect c-Jun phosphorylation at Ser 63/Ser 73

JNK-mediated phosphorylation of c-Jun at Ser 63 and Ser 73 is critical for fibroblast migration (Javelaud *et al.*, 2003). To understand if AOX modulates phosphorylation of c-Jun at Ser 63 and Ser 73, the same set of JNK modulators were tested for their effects on c-Jun phosphorylation at these sites. This was done in iMEFs (Fig. 13 A), as well as in two other cell lines, the HEK AP-1 line used for transcriptional assays (Fig. 13 B) and human fibroblast line BJ-5ta (Fig. 13 C). Only SP600125 decreased the amount of phosphorylated c-Jun, whereas JNK inhibitor V instead increased it, as did PMA. The presence of AOX did not influence c-Jun phosphorylation at these sites in iMEFs (Fig. 13 A), although it potentiated the effect of SP600125 and inhibitor V in an AOX-expressing BJ-5ta cell clone (III, Fig. 13 C).

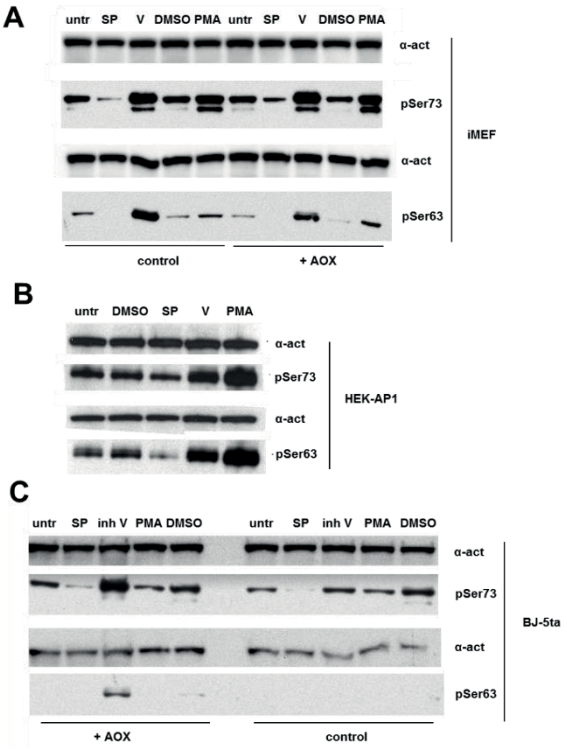


Figure 13. Phosphorylation status of c-Jun Ser 63 and Ser 73 residues. Western blots of cell lysates from (A) control and AOX expressing iMEFs (B) HEK AP-1 cells (C) control or AOX expressing human BJ-5ta fibroblasts. Untreated (untr) or treated with 0.2% DMSO, 20 μM SP600125 (SP) in 0.2% DMSO, 20 μM inh V, or 8 nM PMA. The molecular weights of the bands detected by each antibody were as expected: 100 kDa for α-actinin (α-act), 47 kDa for c-Jun phosphorylated at residues Ser 63 (pSer 63) or Ser 73 (pSer 73). Separate blots were probed for pSer 63 or pSer 73 and reprobed for α-act as a protein loading control. Unmodified from III, Fig. 5.

5.6 AOX expression can influence mammalian cell migration (III)

The failure of thoracic dorsal closure during *Drosophila* development is a defect in cell migration, which AOX expression was able to correct. To test the generality of this finding, I conducted cell migration assays in mammalian cells. MEFs were isolated from AOX hemizygous mice (Szibor *et al.*, 2017) and wt littermates and immortalized using a standard retroviral transduction procedure with viruses encoding human papillomavirus 16 (HPV16) oncoproteins E6 and E7 (Lochmüller

et al., 1999; technical steps conducted by co-authors P.K. Dhandapani and L. Giordano). In these cells, AOX is expressed ubiquitously from an incorporated synthetic CAG promoter, the complete construct being targeted into the ubiquitously active *Rosa26* locus (Szibor *et al.*, 2017).

The drugs SP600125 and AS601245 have both been shown to decrease cell migration (Jarvelud *et al.*, 2003; Cerbone *et al.*, 2012) and were used here for that purpose. Both drugs are potent, reversible, and an (which express AOX at higher level than *tub*AOX and UAS-AOX$^{7.1}$ fly lines)ATP-competitive inhibitor of JNK.

In iMEFs AOX increased the migration rate in all tested conditions in the wound healing essay, except when cells were treated with inh V. The scratch assay conducted on primary MEFs (at passage 6) revealed no difference in migration rate between AOX expressing and control MEFs (Fig. 14 B). All primary lines migrated much slower than iMEFs. Leading edge cells in AOX expressing fibroblasts were able to detach and scout the surface alone, making long protrusions. The wild-type iMEFs leading edge migrated as a whole (data not presented).

To test if the effect of AOX is dependent on cell-cell contact, a single cell migration assay was conducted. The migration speed of single-cell migration of AOX expressing iMEFs was also significantly greater than that of control iMEFs in this assay (Fig. 14 C) indicating that faster cell migration is an intrinsic property of the AOX-expressing iMEFs.

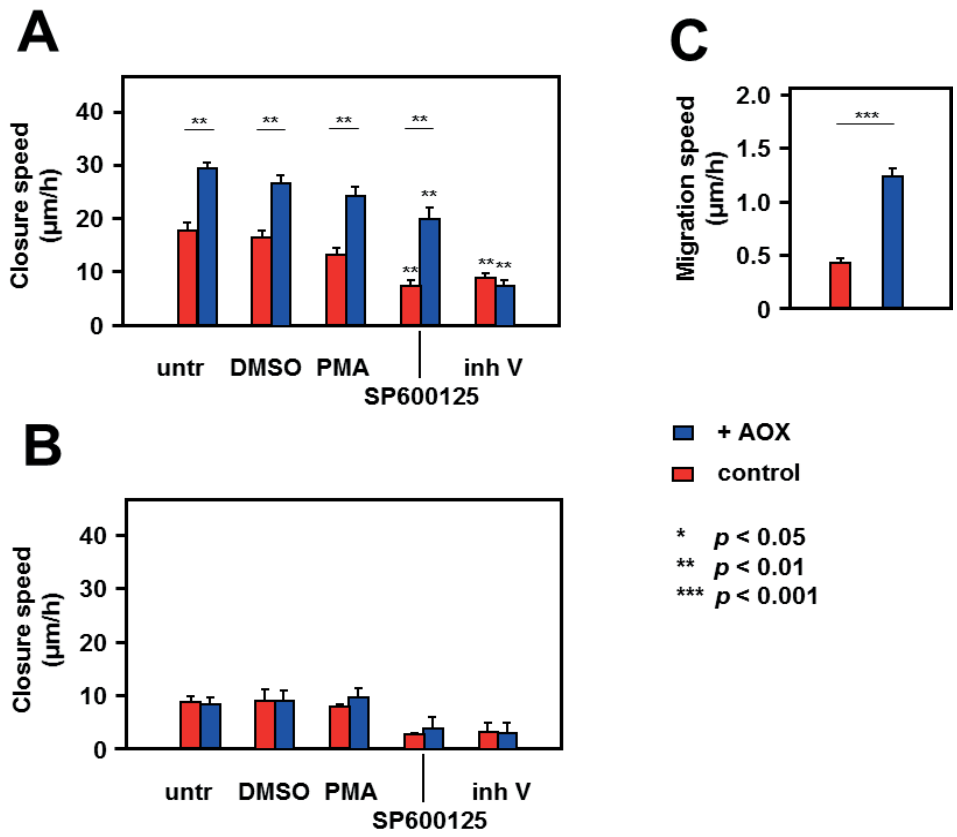


Figure 14. Effects of AOX on mammalian cell migration. (A, B) Wound closure rate of cultured (A) wt iMEFs (control) and AOX hemizygous iMEFs and (B) primary MEFs at passage six, either untreated (untr), or treated with: 0.2% DMSO, PMA (20 nM), SP600125 (20 µM in 0.2% DMSO), inh V (20 µM). Asterisks joining the bars denote statistically significant differences between the genotypes for a given treatment. Asterisks above the bars denote a statistically significant difference from untreated cells of the given genotype. (One-way ANOVA with the Tukey *post hoc* honestly significant difference test). Data represent three biological replicates, each analyzed in triplicate, except for DMSO, which used only two biological replicates. (B) The means ± SD are for pooled data from two cell lines of each genotype analyzed in triplicate at passage six. (C) Single cell migration rate of the iMEFs indicated genotypes. Asterisks denote statistical significance. (Student's *t*-test, unpaired). *n*=31 for control iMEFs; *n*=21 for AOX expressing iMEFs. Unmodified from III, Fig. 4.

5.7 Antimycin A potentiates the migration of AOX-expressing cells (III)

To be able to understand further how AOX influences the migration we tested various metabolic effectors in conjunction with AOX. From various OXPHOS inhibitors, antioxidants and uncouplers, tested, only antimycin A, which binds to quinone reduction site of the cytochrome bc1 complex (Huang *et al.*, 2005), gave differential effect. Antimycin A stimulated the migration of the AOX iMEFs but suppressing the migration of the MEFs (III, Fig. 8 B). To understand these findings we checked respiration in the cell lines tested (III, Fig. 8 C) to observe the effects of AOX under antimycin A. AOX had no significant effect on whole-cell respiration or on permeabilized cell respiration on complex I-, II-, and IV-linked substrates. However, in the presence of antimycin A, AOX enabled almost 80% of the uninhibited respiration rate in permeabilized cells, driven by succinate. This exceeded the measured rate of whole-cell respiration under uninhibited conditions. Capacity for AOX-mediated respiration was sufficient to maintain normal respiratory electron flow in iMEFs in the presence of antimycin A (Experiment conducted jointly with E. Dufour).

6 DISCUSSION

6.1 Overview

This work aimed to test whether transgenically expressed AOX can affect phenotypes related to the impairment of JNK signaling. We have found that expression of AOX was able to promote cell migration in three different models: in *Drosophila*, AOX expression corrected thorax defects caused by activated GS (II) and also when the JNK pathway was attenuated at several specific steps (III). It also promoted cell migration in AOX-expressing iMEFs (III). AOX was not able to correct migration defects when either of the *Drosophila c-fos* and *c-jun* homologues *kay* and *Jra*, respectively, were downregulated, as well as downstream targets of AP-1 such as *pnr* and *puc*. AOX was also unable to enhance the migration of primary MEFs. AOX had no effect on AP-1-dependent transcription in proliferating *Drosophila* (S2) or mammalian (HEK293-derived) cells. The migration of AOX expressing iMEFs compared with that of control iMEFs was differentially stimulated by antimycin A but not by other compounds that affect the mitochondrial respiratory chain, membrane potential, production of ATP or generation of ROS. The summary of the main results from this thesis are illustrated in Figure 15.

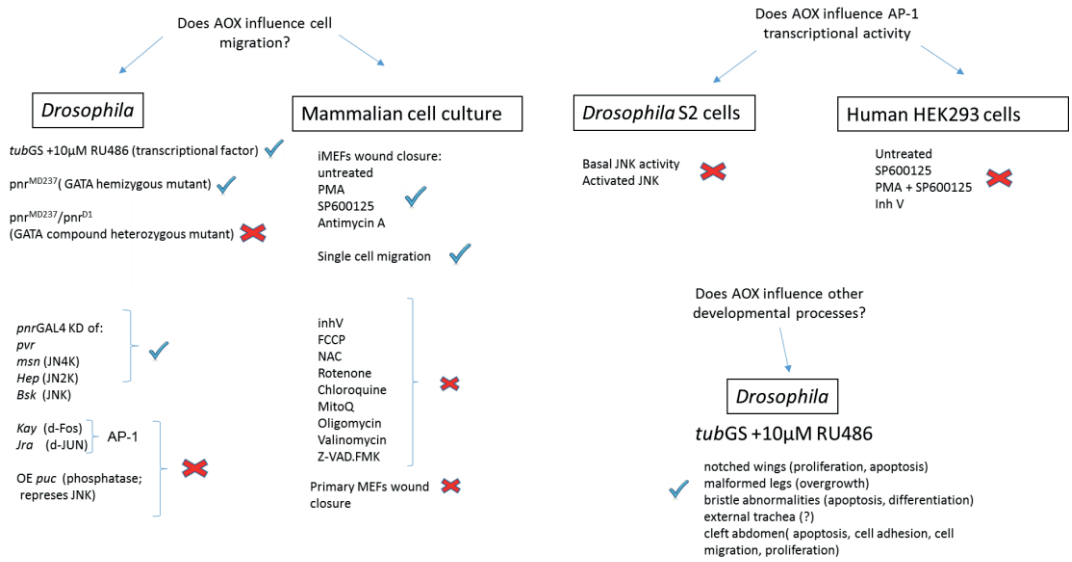


Figure 15. Summary of AOX influences on cell phenotype.

6.2 Use of the mutAOX control

6.2.1 Validation of mutAOX

To validate the use of the catalytically inactive mutated AOX, I first verified that it was targeted to the mitochondria and is compatible with life. I expressed the mutant AOX gene both in mammalian cells and *Drosophila*. Transiently expressed mutAOX in human cells was the same size as AOX (Fig. 6 A) and efficiently targeted to mitochondria of S2 cells (data not presented). MutAOX was shown to lack enzymatic activity using polarography (I, Fig. 4). In a functional assay carried out in *Drosophila*, expression of the mutated enzyme was unable to rescue the organismal phenotypes arising from engineered cytochrome *c* oxidase deficiency (I, Fig. 5).

To create a catalytically inactive protein we chose to mutate four amino acids to alanine: E239A, H242A, E344A, H347A, even though in theory the mutagenesis of a single glutamate residue would be enough to destabilize the di-iron center. In parallel, a catalytically inactive mutant AOX has been engineered by introducing the mutation Y297F, targeting another conserved domain of the enzyme. However, when expressed in human cells the Y297F mutant AOX accumulated to much lower

levels than the wild-type protein, suggesting that this particular mutant protein is unstable and rapidly degraded (P.K. Dhandapani, personal communication). We showed that targeted mutagenesis described in I resulted in stable but catalytically inactive protein.

6.2.2 Enzymatic activity of AOX is important for phenotypic rescue

MutAOX was not able to rescue locomotor defects in the fly arising from engineered cytochrome oxidase deficiency. MutAOX and the low expression AOX line created in (I), showed that rescue of locomotor activity in the COX 7a knockdown fly is dependent on the enzymatic activity of AOX and is related to the amount of AOX expression/activity. The lower dose of AOX was not able to rescue lethality induced by ubiquitous COX knockdown (data not shown), but was able to rescue locomotor defects from neuronal-specific knockdown to a lesser extent than high doses of AOX (I, Fig. 5 B). This narrows down the mechanistic explanations for the rescue of the COX 7a knockdown phenotype (discussed later). This result also validates the usefulness of the fly lines expressing mutant AOX created in (I).

In flies, the mutAOX transgene, introduced into the genome by targeted ΦC31 insertion, showed 3-4 fold lower expression at the RNA level than the AOX transgenic lines previously engineered by P-element insertion (Fig. 6 B, C). Therefore mutAOX *Drosophila* lines generated by ΦC31 insertion are not suitable as controls for those expressing AOX at a high level, since different levels of expression may influence the observed phenotype. This is clearly illustrated in the AOX rescue of GS-induced developmental abnormalities (II, Fig. 5), where the mutAOX-expressing flies were compared with transgenic flies generated in parallel, that expressed wild-type AOX from the same insertion site, i.e. at the same low level. In this case, as well as in the case of AOX rescue of locomotor deficit caused by cytochrome oxidase knockdown (I), lower expression of AOX gave an intermediate phenotype between that produced by wild-type AOX and that seen in non-transgenic control flies. mutAOX gave no such rescue at all. Implementing this control was not possible in the case of JNK knockdown, where I found that low-level AOX expression gave only a marginal or insignificant rescue of cleft thorax (unpublished data). It would thus be useful to create flies with high expression levels of mutAOX to test whether the observed rescue can truly be attributed to the catalytic function of the AOX enzyme. Nevertheless, the creation of ΦC31-engineered fly lines containing: AOX, mutAOX and empty vector at the exact same

position in the genome allows, in principle, for a rigorous experimental setup to understand if AOX-related phenotypes arise from the catalytic action of AOX, or are simply a hormetic effect due to the presence of a xenotopically expressed protein in the IMM. My findings leave open the possibility that AOX has a yet to be defined function or mode of regulation. The *Drosophila* lines that were engineered for this work are currently being used by other researchers who are addressing the dose dependence of AOX in other studies. However, I was not able to use them for the JNK knockdown study for the reasons indicated above, given that all the rescue data were obtained using AOX lines exhibiting a higher level of transgene expression (III).

6.2.3 Level of AOX activity is important for rescue of cleft thorax

In a second example, a single copy of AOX, when constitutively expressed under the α-*tubulin* promoter at a much lower level than when driven by *tub*GS, could not rescue the GS-induced cleft thorax phenotype. However, five doses of the *tub*AOX transgene rescued (II, Fig. 4 C) comparable with the UAS-AOX lines (which express AOX at higher level than *tub*AOX and UAS-AOX$^{7.1}$ fly lines). Once again, I conclude that AOX provides rescue in a dose-dependent manner. This result is consistent with work on mammalian respiratory membranes where, with increasing AOX supplementation to the membranes, the rate of NADH oxidation increases substantially (Fedor & Hirst, 2018). It would be interesting to test the limits of this effect, and whether too high an expression level of AOX is also detrimental to the rescue of cleft thorax. This would imply a 'Goldilocks' effect, perhaps by regulating ROS, which are also known to operate in such a way (Reczek & Chandel, 2017).

As an alternative to the use of mutAOX as a control, it may be possible to use a small-molecule inhibitor of AOX in such studies. Such an inhibitor would have to be specific for the *Ciona* AOX and proven to be harmless to the model system(s) employed. Unfortunately, this is not the case for the known AOX inhibitors n-PG or SHAM.

6.3 *tub*GS induces developmental abnormalities in the fly

Transcription factors act in a coordinated manner to direct cell division, growth, and cell death throughout life. During embryonic development, the coordinated action of transcription factors is also needed to drive cell migration and body plan.

pnr^{MD237} GAL4 driven downregulation of JNK pathway components affects only the notum of the fly (Fig. 9), while GS under the control of the α-*tubulin* promoter is expressed ubiquitously in the fly. I found that it affects the development of various tissues and appendages, leading to cleft thorax, cleft abdomen, externalization of trachea, malformed bristles and notched wings (Fig. 8). A different GS driver, with a much lower and more restricted expression pattern (III, Fig. S2), affected bristle organization but only very subtly. The major dysmorphologies I observed with GS have been reported previously in a variety of mutants, often in combinations similar to this observation. Cleft thorax together with bent and misshaped sensory bristles and severely enlarged legs have been reported in Ultraspiracle mutants (Henrich *et al.*, 1994), a dimerization partner of the ecdysone receptor and one of the key nuclear receptors which regulates fly development. GS is a steroid-activated chimeric GAL4 protein (GAL4-progesterone-receptor fusion protein) (Fig. 3 B). Thus, it is not surprising that GS can affect so many tissues, given that *Drosophila* has such a complex development. Legs are derived from the same ancestor cells as the wing discs in early embryogenesis (Cohen *et al.*, 1993). Tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila*, with differences in their fate controlled by the activation state of the wingless signaling pathway (Franch-Marro, 2006). Tracheal cells in *Drosophila* always arise in close proximity to the cells that are fated to give rise to the legs (Franch-Marro, 2006). The externalized trachea were always localized ventrally, close to posterior leg (Fig. 8 H).

This all implies that GS might act already in early embryogenesis to produce the observed malformations, for example by interacting with ecdysone receptor signaling or other signaling pathways that impinge on JNK signaling in early development. Cleft thorax together with missing or extra bristles has also been reported in mutants of the GATA transcription factor pannier (Heitzler *et al.*, 1996). Cleft thorax is observed when the nuclear transcription factor Y (dNF-Y) is downregulated. This is due to a decreased level of Bsk, as dNF-Y has been found to bind the *bsk* (JNK) gene promoter region, which contains a CCAAT box (Yoshioka *et al.*, 2008). The pair-rule transcription factor gene *odd* regulates the segment polarity genes *en* and *wg* in the early embryo and also restricts JNK signaling to medial edge cells of the peripodial membrane for proper thorax closure. Misregulation of *odd*

leads to thorax closure defects (Tripura *et al*., 2011). Bristle abnormalities similar to those that we observed are also reported in mutants of the gene *u-shaped*, which is a dimerization partner of pannier (Cubadda *et al*., 1997). A completely different way in which GS may affect development is by proteotoxicity, as discussed in a later section (6.5.2).

Whether and how the GS effect is related to JNK signaling still remains a puzzle and unraveling this relationship may shed light on the role of JNK signaling in *Drosophila* development more broadly. It could be that GS interacts with other transcriptional factors at the promoter regions of many developmental selector genes. A chromatin immunoprecipitation (ChIP) assay could be performed to understand whether GS binds in the *Drosophila* genome to the 5´ flanking regions of any of the genes known to influence development.

6.4 Model systems: pros and cons

6.4.1 Limitations of the fly model

The majority of fundamental biological mechanisms and pathways that control development are conserved across evolution between species (Jennings, 2011). This allows the use of simple model organisms, like yeast, nematodes, *Drosophila* or the zebrafish, to study complex molecular processes that occur in the human body. It was Charles W. Woodworth, an American entomologist, who first proposed the use of *Drosophila melanogaster* as a genetic model organism (Sturtevant, 1959). Since Thomas Hunt Morgan's pioneering work in the 1930s, *Drosophila* has been extensively used in the study of animal development and behavior, neurobiology, human genetic diseases and drug discovery. We can test the effects of novel therapies by exploiting genetic tools available in this model, one of which is RNA interference (RNAi) which I used (III) to downregulate the expression of genes involved in thorax formation, leading to defective midline closure. I also used another tool, UAS-GAL4, to overexpress the AOX enzyme from *C. intestinalis*, and observed that this was able to reverse the induced thoracic defect (II, III).

However, *Drosophila* also has limitations. First, although the fly model helped me to understand which downregulated genes AOX can compensate for, and build a testable hypothesis, it was not possible to use it to elucidate the mechanism by which AOX provides this rescue. Techniques to isolate the specific cells where the JNK

pathway was downregulated and test my hypothesis are not currently available. This would require isolation of the limited cell population where JNK is active during the larval and pupal stage from the progeny containing all the needed transgenes for downregulation of the gene and rescue. There is currently no way to distinguish these larvae or pupae from controls, due to the specificity of the crosses and lack of markers. Validating my findings (III) in mammalian cells allowed me to test hypothetical AOX rescue mechanism(s) in a more experimentally malleable system that also has direct translational relevance to human disease. The fact that AOX was found to promote migration in both models, supports but does not prove that it acts by a common mechanism, whether by ROS dampening, heat production or something else.

6.4.2 Developmental disturbance in *Drosophila* resulting from the GS system

Drosophila is an invaluable model system in research because of its vast and easy to use genetic toolkit, providing over-expression, knockdown, and knockout of particular genes. Also, 75% of *Drosophila* genes have counterparts in the human genome (Pandey & Nichols, 2011). However, we are not always fully aware of the limitations of the tools available for research. To spatially and temporally control gene expression in a living organism is invaluable, but even an elegant system such as GeneSwitch in *Drosophila* has potential flaws that must be considered.

The use of the GS system assumes that it is inert in the fly. However, the findings reported here suggest otherwise. I used *tub*GS and *da*GS drivers to ubiquitously drive the expression of AOX transgenes during development (II). I identified specific developmental abnormalities arising from the use of *tub*GS in combination with RU486, including substantial pupal lethality already at 2.5 μM RU486, and from 10 μM dysmorphologies described above (Fig. 7 and 8). This suggests that the *tub*GS system itself induces such defects, by affecting transcription as hypothesized above. The chimeric GS protein should be inert, as there is no UAS sequence in the *Drosophila* genome to which it can bind. However, Liu & Lehmann (2008) showed that expression of high levels of the Gal4 protein in *Drosophila* can cause a genomic response, where modified expression was found, of many genes involved in development. Since GS is a modified Gal4 protein, it is perhaps unsurprising that it induces developmental defects (II), and conversely surprising that this has been previously ignored.

My work raises concerns about the use of the *tub*GS system. To avoid misinterpreting data it is advisable to use it in conjunction with control experiments that carefully monitor the effects of activated GS alone. We cannot rule out that there are other disturbances when using the system, even with other GS drivers and at lower RU486 concentrations than used here. Thus, all studies should control for the effect of the transcription factor plus RU486 alone, when drawing conclusions. There are many studies (e.g., Myllymäki & Rämet, 2013; Cho *et al.*, 2014; Kim *et al.*, 2015) that may have reached misleading conclusions due to a lack of such controls.

6.4.3 Limitations of the cell model

One intriguing issue that arose was the difference in sensitivity to AOX between immortalized and primary MEFs (Fig. 14 A, B). In fact, it was difficult to perform wound healing assays on primary MEFs. Primary MEFs were extremely sensitive to JNK inhibitors, which iMEFs handled without observable problems. The use of human fibroblasts was set aside during the study, after it was discovered that the AOX-transduced BJ-5ta cell line exhibited structurally impaired mitochondria with decreased complex I activity (data not presented). Further investigation using other cell-lines would be needed, to confirm the generality of the effects of AOX on cell migration, and the relationship to immortalization or transformation.

6.4.4 Limitations of the scratch-wounded confluent monolayer of fibroblasts

During re-epithelialisation of the epidermis after injury, cells at the leading edge are able to undertake cytoskeletal changes; extending lamellipodia, filopodia and polarizing in the direction of migration (Wager *et al.*, 2017). Individual cells can take on a leading role in collective migration, forming a cell group termed 'leader cells' that have a specific phenotype and profile of gene expression independent of other cells (Wager *et al.*, 2017; Khalil & Friedl, 2010; Riahi *et al.*, 2012). In a simple 2D system, a technique which recapitulates cutaneous wound re-epithelialisation (Wager *et al.*, 2017), I observed that the AOX-expressing wound healing monolayer has single 'leader cells', which were able to detach from other cells and explore the environment, making more protrusions than non-AOX control cells (data not presented). Computational methods could be applied to this data to extract different parameters than those used conventionally to describe collective cell migration. 2D and 3D environments are also different in terms of cell adhesion and migration

behaviors (Vu *et al.*, 2015). *In vivo*, cells move in a complex environment defined by a combination of ECM proteins, proteoglycans, polysaccharides, growth factors, and signaling molecules (Frantz *et al.*, 2010). Here, I tested the effect of AOX on the migration of cells in a purely 2D assay, stripped from any influence of the ECM. It would be interesting to test whether AOX can promote cell migration in models that better mimic the cellular environment *in vivo*, such as 3D scaffolds manufactured using biomaterials (Yang *et al.*, 2017) or organoid models, e.g., the organotypic culture model of mammary branching morphogenesis (Ewald *et al.*, 2008) before the wound healing is attempted in mice.

6.4.5 Limitations of using GFP as a control

Expression of an exogenous protein can perturb cell structure and function in unpredictable ways. This issue is inherent to any experiment, and is clearly demonstrated by my findings in the case of the GS system (II). This was why I set out to create the catalytically inactive variant of AOX (I), which unfortunately was not available at the start of the JNK study, and anyway proved to be an insufficiently strong expressor. Instead, I initially used GFP expressed in the cytoplasm as a routine control in all AOX expression studies (II, III), which has its own limitations. GFP is a structurally and functionally distinct protein from AOX, thus having different properties when expressed as a foreign protein in cells. Even though it is considered an inert protein in model systems it has been shown that wild-type GFP from the jellyfish, *Aequorea victoria*, quenches $O_2^{\bullet-}$ and has SOD-like activity by competing with cytochrome *c* for $O_2^{\bullet-}$ (Bou-Abdallah & Chasteen, 2006). However, since GFP as used in my experiments resides in the cytosol, it should not have access to mitochondrially generated superoxide, nor should it interact with or compete with cytochrome *c*. On the other hand, the fact that it is expressed in a separate cellular compartment than AOX, which is an inner mitochondrial membrane protein, means that it may have different non-specific effects on cellular protein homeostasis. Nevertheless, by modulating ROS or interacting with cytochrome *c*, AOX could influence apoptosis during development, which could impact the mechanism behind any rescue. AOX acts by oxidizing quinol and preventing cytochrome *c* from reduction, while GFP may restrict cytochrome *c* oxidation. Thus, they might be predicted to have opposite effects, which I indeed observed when attempting some instances of JNK-pathway rescue with AOX and GFP (III, Fig. 3 F). The JNK

signal-transduction pathway appears to be essential for the execution of apoptosis in response to several different stimuli (Davis *et al.*, 2000). Apoptosis is needed in pupae where imaginal cells undergo apoptosis during thorax formation (Martín-Blanco *et al.*, 2000 (Fig. 2). In the case of JNK knockdown AOX could be instrumental in 'replacing' JNK by promoting apoptosis, for which GFP is not so much a control as a parallel experiment.

6.5 Possible mechanisms of AOX rescue of cell migration defects

6.5.1 AOX rescue depends on its enzymatic activation

Based on my findings (I, II), the effects of AOX on *Drosophila* depend upon on its activation, at least in the case of locomotor impairment resulting from COX 7a knockdown (I) and the developmental abnormalities resulting from activated *tub*GS (Fig. 7). This is also most likely the case for rescue of JNK knockdown and promotion of cell migration in the wound-healing assay (III). Data obtained from many transgenic models, including human cells (Hakkaart *et al.*, 2006; Dassa *et al.*, 2009), mice (El-Khoury *et al.*, 2013) and flies (Fernandez-Ayala *et al.*, 2009) indicate that the AOX from *C. intestinalis* becomes activated only when needed, i.e. when the cytochrome pathway is blocked, and there is resulting over-reduction of the quinone pool. This is consistent with previous studies in other organisms (Hoefnagel & Wiskich, 1998; Castro-Guerrero *et al.*, 2004), which showed that AOX activation occurs when ubiquinol accumulates to substantial levels due to respiratory chain inhibition or overload. This would mean that AOX activation during development, when the JNK pathway is inhibited or if GS is active could be due to mimicking this condition. However, not much is known about the activation of AOX in metazoans, leaving open the possibility that the downregulation of JNK signaling or the presence of activated GS leads to the enzymatic activation of AOX by an as yet unknown metabolic mechanism. What we know is that AOX activity in plants increases with cold (Stewart *et al.*, 1990) or oxidative stress (Wagner, 1995). This further implies that there might be disturbances in the redox state of the developing cells of the thoracic epithelium due to JNK downregulation or GS activation, or in a cell monolayer responding to wounding.

6.5.2 AOX decreases ROS

It is known that AP-1 mobilizes oxidative defense and it is at the same time sensitive to oxidation and therefore protected by number of proteins. For example, redox factor 1 (Ref-1) is involved in reduction of the critical cysteine residues of Fos and Jun to maintain their DNA-binding activity following oxidative stress (Xanthoudakis & Curran, 1992). MBF1 also protects the critical cysteine residues from oxidation (Jindra *et al.*, 2004) and stimulates AP-1 binding to DNA. AOX could be instrumental in protection of AP-1 from oxidation by generally decreasing mtROS when activated (Dogan *et al.*, 2018). This, however needs to be tested by measuring ROS in the relevant cells, and assessing the status of the cysteine residues of AP-1 by redox proteomics. However, I could not see any change in AP-1 transcriptional readout in AOX-expressing S2 cells, nor in mammalian cells when JNK was modulated. This implies that the mechanism of AOX action is not due to ROS. Furthermore, this conclusion is supported by the lack of any effect of ROS modulators on the AOX-dependent stimulation of migration in the wound-healing assay (III, Fig. 8).

6.5.3 AOX is a thermogenic protein

Temperature is what Richard Feynman called the 'jiggling' of atoms (Sengupta & Garrity, 2013). Thermosensors are molecules whose temperature sensitivity serves as information about the thermal environment and trigger an appropriate physiological or behavioral response (Sengupta & Garrity, 2013). What that means is that temperature alters the configuration of the atoms of biomolecules, affecting their activity. Environmental temperatures and their changes can be read by nucleic acids, lipids and proteins, acting as thermosensors, which then regulate cellular processes, including transcription, protein stability and signal transduction (Sengupta & Garrity, 2013). Such processes could underlie the effects of AOX.

When AOX is active, electrons are passed to oxygen without proton pumping, so that the energy of the reaction is used to produce heat. It is well documented that AOX is thermogenic in plants under specific circumstances; for example in the flowers of *Philodendron selloum* and *Symplocarpus foetidus*, which can heat up to 35 °C above ambient temperature (Elthon & Mcintosh, 1987; Wagner *et al.*, 2008; Seymour,

2000). We showed in a recent report that *Ciona* AOX expressed in *Drosophila* may be functionally thermogenic under specific physiological conditions, providing cold resistance to developing and adult flies (Saari *et al.*, 2018). Consequently, if enzymatically active, AOX could increase the temperature of the developing thorax in *Drosophila*, which in turn may activate or by-pass JNK signaling by a variety of mechanisms, allowing migration to proceed. One such mechanism could simply be a thermogenic potentiation against oxidative stress, strengthening JNK signaling (Courtial *et al.*, 2017). Increased phosphorylation of JNK was observed in human fibroblasts under thermal stress (Courtial *et al.*, 2017). Another mechanism could involve heat-shock proteins. Heat shock protein 90 (Hsp90) has been reported to stabilize proapoptotic JNK signaling (Nieto-Miguel *et al.*, 2007), whilst heat shock protein 70 (Hsp70) has been shown to promote cell migration by acting as a chaperone to deliver proteins to the leading edge (Boroughs *et al.*, 2011).

As discussed above, a likely way in which GS influences development in *Drosophila* is via transcription. However, an alternate mechanism could be its accumulation in the cell causing proteotoxicity. The latter hypothesis is tentatively supported by the fact that I observed the same developmental abnormality, cleft thorax, when the human huntingtin protein with an extended polyglutamine repeat of 93 residues (Httex1p Q93) was ubiquitously expressed in *Drosophila* (unpublished data). A proteotoxic effect could potentially be mitigated by the fact that AOX being thermogenic activates the heat-shock response, which is instrumental in decreasing misfolded proteins, thus providing the rescue. It would be interesting to attempt AOX rescue of the cleft thorax induced by Httex1p Q93.

6.5.4 AOX affects ATP production

AOX activation would mean decreased electron flow through complex III and cytochrome *c* oxidase and thus, decreased ATP production. This might be exacerbated by increased heat production. These changes could provide the initial stimulus for JNK activation as a stress response. However, treatments that limit mitochondrial ATP production (oligomycin, FCCP and rotenone) did not have differential effect on the migration of AOX-expressing cells in the wound healing assay (III, Fig. 8) showing that ATP modulation is most likely not behind the mechanism of AOX action in that context. In the fly, AOX rescued cleft thorax induced by JNK knockdown, and in iMEFS restored migration when JNK was

blocked by SP600125. However, despite their apparent similarity, these two effects of AOX could be via different mechanisms.

6.5.5 Potential interplay between AOX and mitochondrially localized JNK

JNK functions as a signal transducer that conveys cytosolic oxidative stress signals to both the nucleus and mitochondria. JNK phosphorylates c-Jun, p-53, Elk-1, ATF-2 (Johnson & Nakamura, 2007; Weston & Davis, 2007) as well as Bax protein, promoting apoptosis (Bong-Jo *et al.*, 2006). JNK translocates to mitochondria and, by facilitating cytochrome *c* and SMAC release from the intermembrane space, it can induce apoptosis (Aoki *et al.*, 2002). JNK also phosphorylates pyruvate dehydrogenase (PDH). It has been proposed that JNK kinase may trigger either a phosphorylation cascade across mitochondrial membranes involving unidentified intermediates, or activate a second messenger, such as Ca^{2+}, leading to the activation of PDH (Zhou *et al.*, 2008).

Mitochondria are sources of H_2O_2 and NO (Cadenas & Davies 2000; Poderoso *et al.* 2000). Antimycin A increases H_2O_2 production due to its action on complex III, which in turn results in the translocation of JNK into the mitochondria (Hanawa *et al.*, 2008). The potentiation of cell migration in AOX-expressing iMEFs, but not in the control iMEFs suggests that proximity of JNK when complex III is blocked creates conditions for the AOX to promote cell migration.

6.5.6 AOX and IMM shape

The shape of the inner mitochondrial membrane influences mitochondrial function (Mannella, 2006). For example ATP synthase contributes to the curving of the IMM as result of its oligomerization (Paumard *et al.*, 2002; Strauss *et al.*, 2008). Optic atrophy 1 (OPA1) protein is anchored to the IMM and is another protein involved in mitochondrial shape which affects its functions (Campello & Scorrano, 2010; Belenguer & Pellegrini, 2013). New mitochondria-shaping proteins influencing their function continue to be discovered.

The presence in the IMM of the *Ciona* AOX protein, which presumably functions as a dimer, might affect inner membrane organization which could have various bioenergetic implications. Altered IMM topology might affect mitochondrial signaling leading to the activation of JNK or of a parallel pathway. However, whilst

this might be a plausible mechanism to account for the promotion of iMEF migration by AOX, it cannot explain why AOX corrects cleft thorax and other developmental abnormalities caused by *tub*GS plus RU486, since this requires the enzymatic activity of AOX, as shown by the mutAOX control (II).

6.6 Potential use of AOX in therapy

Despite sequence conservation, AOXs of different species exhibit considerable diversity in enzymatic properties (May *et al.*, 2017). How *Ciona* AOX is regulated is still unclear, and there is insufficient data on animal AOX in general to address this issue (McDonald *et al.*, 2009). AOX expressed in animals possess a unique C-terminal of unknown function (McDonald *et al.*, 2009), but also lack an N-terminal cysteine residue which is important for enzyme regulation in plants (McDonald *et al.*, 2009). In plants, AOX protein is stress-regulated (as well as being tissue-specific): for example, it is dramatically increased within 5 hours of addition of antimycin A in tobacco (Vanlerberghe & Mclntosh, 1992). Our own recent work shows that AOX is induced in *Ciona* by hypoxia and sulfide exposure (Saari *et al.*, 2018). Obviously, such regulation is lost in transgenic models, unless it operates post-transcriptionally. Conversely, the inference that AOX is activated in specific contexts in *Drosophila* development and in migrating mammalian cells may provide a handle on understanding exactly how and by what the enzyme is activated metabolically, which could help assess better how to use or engineer it therapeutically. In this context, it would be illuminating to test AOXs from other organisms (e.g., plants, fungi or trypanosomes) in the same assays used here.

A similar type of epithelial sheet movement to that seen in thoracic closure in *Drosophila* occurs in ventral closure in *C. elegans*, and in neural tube closure, embryonic wound healing and epiboly in vertebrates (Harden, 2002). In addition, key events in cancer progression show remarkable similarities with wound healing (Chang *et al.*, 2004), suggesting that cancer involves deregulation of normal wound healing processes (Dauer *et al.*, 2005). DNA microarray analyses have shown that the gene expression pattern of healing skin resembles that of malignant tumors (Iyer *et al.*, 1999; Chang *et al.*,2004). Wounds which do not heal could be a risk factor for malignant transformation (Schäfer & Werner, 2008) due to specific changes in gene expression (Chang *et al.*, 2004). This strengthens the idea proposed by Dvorak (1996) that "tumor stroma generation is wound healing gone awry." Accelerated wound

healing is desirable, e.g., in victims of burns, and in people with slow-healing skin lesions as seen in diabetics (Greenhalgh, 2003) or following surgery, At the same time we do not want wound healing to go awry, promoting tumor formation.

Once the mechanism is more clearly established, a follow-up study would be to test AOX in a mouse wound-healing model (Dunn *et al.*, 2013), aiming eventually to apply the knowledge in human diseases such as chronic wounds, where the underlying biology is still not well understood (Nuutila *et al.*, 2014). Translating between models is appropriate in this case because the molecular machinery and mechanisms driving wound healing resemble those found in tissue fusion events during animal development (Kurosaka & Kashina, 2008), including dorsal and thoracic closure in the *Drosophila* embryo. Based on my findings, AOX could also be applied therapeutically in humans to prevent developmental abnormalities; however, its use in any application could also promote tumor invasion. The outcome may depend on the specific context of where, when and how much AOX is being expressed. AOX may therefore prove most useful as a tool to elucidate the underlying processes and ensure a safe outcome in a wide variety of interventions.

7 CONCLUSIONS

This is the first report that xenotopic expression of AOX remedies developmental abnormalities that occur without any documented mitochondrial respiratory chain defects. That itself raises the intriguing question of how alterations to nuclear-receptor and JNK signaling may influence mitochondria and *vice versa*. Using data obtained from two tested models: thorax closure in *Drosophila* and wound healing in mammalian cells, I am able to propose that mitochondria impact collective cell migration, specifically involving the respiratory chain at the level of complex III and/or IV. Perturbation of the mitochondrial RC affects signaling pathways which control cell migration. The importance of this discovery which combines *in vivo* model organism experiments and *in vitro* cell culture should lead to further studies investigating the mechanism whereby mitochondrial respiratory function affects cell migration and other developmental programs. My observation that AOX influences the migration of iMEFs, which are highly dependent on glycolysis, but not that of primary MEFs, suggests a new dimension in considering the links between mitochondrial metabolism and cancer metastasis. Other important questions arising from my work concern the role of ROS and mitochondrial heat production in regulating the intracellular signals responsible for lamellipodia formation, actin cytoskeleton remodeling and focal adhesion turnover. AOX is believed to act, indirectly and perhaps also directly, as a scavenger of mitochondrially generated ROS; but it is also thermogenic. Although a unifying hypothesis would be attractive, there could be different modes of action of AOX affecting cell migration in these different models. A temperature effect could operate in one case, but ROS modulation in another. Using an omics approach could give insight into possible mechanisms of AOX action which can then be subjected to more rigorous experimental testing. Omics could also be used to probe how GS causes such a wide spectrum of malformations in *Drosophila*, and how this relates to interference with transcription, cell signaling or proteotoxic stress.

The development of *in vivo* thermal probes for use in *Drosophila* would enable measuring the temperature in the pupa during thorax formation. Indirectly we could test expression of the heatshock proteins, some of which are known to promote cell migration, e.g., Hsp70 binds the protein cross-linking enzyme tissue

transglutaminase (tTG) localizing it to the leading edge in cancer cell migration. (Boroughs *et al.*, 2011). To test the involvement of ROS, we could attempt the rescue of the cleft thorax with catalase or expose flies to agents such as mitoquinone mesylate (MitoQ) or N-acetyl cysteine (NAC).

A full understanding of the mechanism whereby AOX impacts development can potentially enable the design of new treatments for tissue injuries, metastatic tumors and congenital midline closure defects, as well as more 'conventional' effects of mitochondrial dysfunction.

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10 PUBLICATIONS

PUBLICATION I

Diiron centre mutations in *Ciona intestinalis* alternative oxidase abolish enzymatic activity and prevent rescue of cytochrome oxidase deficiency in flies

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Diiron centre mutations in *Ciona intestinalis* alternative oxidase abolish enzymatic activity and prevent rescue of cytochrome oxidase deficiency in flies

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The mitochondrial alternative oxidase, AOX, carries out the non proton-motive re-oxidation of ubiquinol by oxygen in lower eukaryotes, plants and some animals. Here we created a modified version of AOX from *Ciona instestinalis*, carrying mutations at conserved residues predicted to be required for chelation of the diiron prosthetic group. The modified protein was stably expressed in mammalian cells or flies, but lacked enzymatic activity and was unable to rescue the phenotypes of flies knocked down for a subunit of cytochrome oxidase. The mutated AOX transgene is thus a potentially useful tool in studies of the physiological effects of AOX expression.

The mitochondrial alternative oxidase, AOX, carries out the non proton-motive re-oxidation of ubiquinol by molecular oxygen. Terminal electron transfer by AOX constitutes a parallel system to that provided by OXPHOS complexes III and IV in plants, fungi, protists and many animal phyla[1]. AOX is believed to become activated under stress conditions, when the OXPHOS cytochrome chain is overloaded or unavailable.

In many organisms this is achieved, at least in part, via the regulated expression of the AOX gene, which is induced by a variety of stresses relevant to OXPHOS dysfunction[2,3]. The enzyme is also inherently responsive to the metabolic signature of such stresses in different organisms. Firstly, it is activated by high levels of its reduced substrate, ubiquinol[4,5], which is assumed to reflect a lower affinity for the substrate than that exhibited by OXPHOS complex III, with which it competes. Thus, under normal physiological conditions, most of the electron flow from ubiquinol to oxygen is channelled through complexes III and IV, even if AOX is physically present. Only if ubiquinol levels increase, for example, if the enzymatic capacity of complexes III and IV becomes limiting, will AOX become functionally significant. In addition, AOX is allosterically activated in many organisms by metabolites whose levels increase under conditions of OXPHOS insufficiency, for example pyruvate[3], as well as by other metabolites indicative of cellular redox state.

Although the AOX gene has been lost, during the course of evolution, in the lineages leading to the most complex and advanced metazoan groups, including mammals[1], we reasoned that its reintroduction by transgenesis should enable such animals to buffer many of the pathological stresses resulting from OXPHOS dysfunction[6]. Thus AOX could become a therapeutic tool for treating mitochondrial diseases and other conditions mediated by OXPHOS dysfunction[7]. Preliminary tests in model organisms, including cultured human cells[8,9], *Drosophila*[10,11] and the mouse[12], support this concept. In particular, the expression of AOX from the tunicate *Ciona intestinalis*, was shown to compensate many of the phenotypes resulting from cytochrome oxidase (COX, complex IV) deficiency in *Drosophila*, including the knockdown of structurally essential subunits of the complex[11]. However, if AOX is to be of value in eventual therapy, the mechanism of this compensation needs to be established. The hypothesized

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enzymatic by-pass is only one of several possible such mechanisms. Expression of an inert transgene, such as GFP, in place of AOX, was unable to rescue the phenotypes produced by engineered deficiency of cytochrome oxidase[10,11]. However, this control cannot be unambiguously interpreted, since the expressed GFP was not targeted to mitochondria, and even if it were, does not possess other structural features of AOX that enable it to insert into the inner mitochondrial membrane in a specific fashion and interact with other components thereof.

In order to provide a more applicable test of whether the ability of AOX to rescue COX deficiency depends on its primary enzymatic activity, we sought to engineer the AOX in such a way as to destroy this activity, whilst producing only a minimal effect on the overall structure, stability and expression of the protein. To do this, we took advantage of the fact that AOX is well conserved phylogenetically, that the residues contributing to its active site have been characterized in a number of species, and that the structure of a representative AOX, from the protistan parasite *Trypanosoma brucei*, has recently been published[13]. Using currently available bioinformatics tools, we modelled the structure of the *Ciona intestinalis* enzyme against this template, predicted amino-acids required for binding the catalytically essential diiron moiety at the active site, and proceeded via alanine-substitution mutagenesis to create an expressible version of the enzyme expected to lack enzymatic activity, despite being predicted to fold to a similar overall structure. In several different contexts (cultured human and *Drosophila* cells, as well as whole animals), we found that the mutated AOX was stably expressed but devoid of detectable enzymatic activity. Furthermore, expression of the transgene encoding the mutated AOX was unable to rescue engineered COX deficiency in the fly, confirming that this rescue indeed depends on the enzymatic activity of AOX.

Materials and Methods

Sequence alignments and molecular modelling. The sequences of AOX homologues found by BlastP searching were aligned using the MUSCLE algorithm built into the software MEGA6[14], with default parameters. A homologous model of the structure of one subunit of the *C. intestinalis* AOX was generated using the software I-TASSER[15], based on the crystal structure of the *Trypanosoma brucei* AOX (PDB 3VV9:A)[13] as template and the multiple sequence alignment described above as input restraint. Other parameters were set as default. Selection of the model was based upon the best accuracy estimations provided by the C-scores, estimated TM-scores and RMSD values. Because the N-terminal region (M1-K103) of the *C. intestinalis* AOX structure could not be modelled with high accuracy, this region was eliminated from the analysis. The dimeric model of *C. intestinalis* AOX and the positioning of the two diiron centres (one per subunit) were built by overlapping two copies of the model generated by I-TASSER into the crystal structure of the dimeric *T. brucei* AOX using Pymol (www.pymol.org). Pymol was also used to analyze all structure models and to produce the figures.

Cloning procedures and mutagenesis. For *Drosophila* expression, the *C. intestinalis* AOX coding sequence, including its natural stop codon, was recloned from the pMT/V5-His B vector (Invitrogen), in which it had been previously propagated, into the *Eco*RI site of pUASTattB[16]. Based on the multiple sequence alignment shown in Fig. S1, and the results of molecular modelling (see Results), PCR-based alanine substitution mutagenesis and recloning were carried out according to the scheme of Fig. S2. Mutations E239A, H242A, E344A and H347A were introduced, using the plasmid-borne AOX cDNA as template, Pfu DNA polymerase (Stratagene) and oligonucleotides (all shown 5′ to 3′) as follows: GAAGCTGAAAATGcGAGAATGgcCTTAATGACTGCG and CGCAGTCA TTAAGgcCATTCTCgCATTTTCAGCTTC to create E239A/H242A, followed by ATCTGAGCTGAT GcAGCACATgcCAGATCAGTCAAC and GTTGACTGATCTGgcATGTGCTgCATCAGCTCGGAT to create E344A/H347A (lowercase letters indicate the sites of introduced mutations). For expression in S2 cells, constructs containing the original and mutated AOX cDNA inserts, again using the natural stop codon, were recloned into the *Eco*RI site of pAc5.1/V5-His B (Invitrogen, USA) to create pAC/AOX[17] and pAC/mutAOX. For transient mammalian expression, the wild-type and mutated AOX coding sequences were recloned, respectively, into a pBR322-derived *kan*^{R} plasmid containing the CAG promoter[18] and bovine growth hormone poly(A) signal, together with other elements not relevant to the present study (copies of the *tet* operator, *loxP* sites, insulator elements and portions of the porcine *Ggta1* gene), to create the expression constructs pCAG-AOX and pCAG-mutAOX. The nucleotide sequences of all clones were confirmed by Sanger sequencing using the Big Dye Terminator v3.1 kit (Life Technologies) and an ABI3130xl Genetic Analyzer, according to the manufacturer's specifications.

Drosophila stocks and maintenance. Except where stated, flies were maintained and grown on standard medium at 25°C, using a 12 h light/dark cycle, as previously[10,19]. Balancers, recipient line w^{1118}, the RNAi line for CG9603 (Vienna Drosophila RNAi Center line 106661), the ubiquitous *da-GAL4* driver (Bloomington line 8641) and the driver line bearing $elav^{C155}$*-GAL4* on chromosome X and UAS-*Dcr2* on chromosome 2 (Bloomington line 25750), were obtained from stock centres. ΦC31 recombinase-mediated-site-directed transgenesis was used to generate transgenic fly lines (service provided by BestGene Inc, Chino Hills, CA), using recipient lines with the following integration sites: *attP18* (chromosome X), *attP40* (chromosome 2) and *attP2* (chromosome 3), according to Pfeiffer *et al.*[20], employing the wild-type and mutated AOX constructs cloned in pUASTattB and pUASTattB itself as empty-vector control. Following characterization, transgenic lines were maintained over balancers appropriate for chromosome X, 2 or 3, bearing standard markers (FM7, CyO, TM3Sb, respectively). Transgenic lines UAS-AOX^{F24} and UAS-AOX^{F6} were described previously[10].

Cell culture and transfection. HEK293T cells were cultured as previously[21]. Plates of 3×10^6 cells were transfected with 24 μg of the pCAG-AOX or pCAG-mutAOX plasmids or, as control, empty vector (pWPI, Addgene), using 60 μl Lipofectamine® 2000 (Invitrogen) under manufacturer's recommended conditions. *Drosophila* S2 cells were grown and transfected with pAc5.1/V5-His B or derivatives as previously[17].

Expression assays. RNA extraction and QRTPCR to measure AOX transcript levels using *RpL32* RNA as an internal normalization standard were as previously described[10], using RNA from 2 day-old adult male and female flies. Protein extraction from 2 day-old *Drosophila* adults and Western blots were conducted essentially as by Fernandez-Ayala *et al.*[10], with the following modifications: for females, 1% SDS was used for lysis instead of 1.5% Triton X-100, flies were processed in batches of 30 (females) or 40 (males), SDS-PAGE used Any kD™ Criterion™ TGX™ 18-well gels (Bio-Rad), Prestained Protein Ladder (Thermo-Scientific) and ProSieve™ EX Running and Transfer Buffers (Lonza), and membranes were treated in PBS-Tween® instead of TBS. Primary antibodies used were customized rabbit anti-AOX[10] (21st Centrury Biochemicals, 1:10,000), rabbit anti-α-actininin C-20-R (Santa Cruz Biotechnology, 1:5,000) and mouse anti-ATP5A (Abcam, 1:50,000). Secondary antibodies were Peroxidase Goat Anti-rabbit IgG and Horse Anti-mouse IgG (both from Vector Laboratories, 1:10,000). Post-nuclear extracts (PN) from HEK293T cells were prepared according to Cannino *et al.*[21]. Protein concentrations were measured using the Bradford assay.

Respirometry. Oxygen consumption of 5×10^6 human cells was measured 48 h after transfection, following permeabilization with 80 μg/ml digitonin, in a Clark-type electrode (Hansatech Oxytherm system) using respiratory buffer A[22] at 37 °C. Complex II-driven respiration was measured in the presence of 10 mM ADP and 10 mM succinate. AOX-driven (antimycin-resistant) respiration was measured after the further addition of (60 ng/ml) antimycin A, with subtraction of any residual oxygen consumption after adding 100 μM *n*-propyl gallate. Respirometry on S2 cells was as described previously[17] and was also conducted on homogenates from 1–4 day-old *Drosophila* males. Briefly, 25 males were gently homogenized in 0.8 ml ice-cold isolation buffer (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4) and muslin-filtered. Respirometry was performed on 150 μl aliquots of this homogenate, mixed with 500 μl assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES-KOH, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% BSA, pH 7.2), substrates (15 mM glycerol-3-phosphate and 5 mM ADP) and inhibitors as for permeabilized mammalian cells.

Behavioural assays. Time to eclosion following *Drosophila* crosses was measured as previously[23]. Eggs from parents crossed two days earlier were collected over three consecutive nights, and cultured at 25°C. Adults less than 24 h old were collected and sorted on ice, after which batches of 5 male flies were placed in each empty vial. After a 10 min waiting period, flies were tipped down and their subsequent behaviour recorded using a DFK 21AF04 camera (The Imaging Source, Bremen, Germany) and Media Recorder 2 software (Noldus, Wageningen, Netherlands). The climbing index[11] for each vial was manually calculated from recordings as the mean number of flies which climbed 6 cm in 10 s in three trials. Climbing indices from different genotypes were compared by one-way ANOVA with Bonferoni adjustment, using SPSS 12. The box plot was drawn with BoxPlotR (boxplot.tyerslab.com), with Tukey style whiskers extending to the data point that is no more than 1.5 × IQR (interquartile range) from the edge of the box[24].

Human subjects. The work reported here did not use human subjects or any materials derived from human subjects, other than the freely available cell-line HEK293T.

Results and Discussion

Modelling and creation of mutated AOX transgene. Alignment of the predicted *Ciona intestinalis* AOX amino-acid sequence with the corresponding protein from other taxa, including *Trypanosoma brucei*, revealed conservation of residues implicated in the organization of the diiron centre of the enzyme, as previously reported by Shiba *et al.*[13]. The four invariant glutamate residues and two histidines correspond in *Ciona* AOX with E200, E239, E290, E344, H242 and H347 (Fig. S1), numbered from the first methionine of the putative preprotein. In the *Trypanosoma* AOX structure, the conserved histidines participate in a hydrogen bond network that also includes a conserved tyrosine, Y297 in *Ciona* AOX (Fig. S1). Structural modelling (Fig. 1) showed that *Ciona* AOX can fold to an almost identical structure as its *Trypanosoma* counterpart, ignoring the poorly conserved N-terminal region (residues 1–103 of the *Ciona* protein, Fig. S1). Four alpha-helices enclose the diiron centre of each protomer of the homodimeric protein, with the conserved glutamate and histidine residues similarly juxtaposed as in the *Trypanosoma* protein (Fig. 1). Based on this structure, we tested the functional significance of the conserved residues at the predicted diiron centre, by mutating four of them to alanine (E239A, H242A, E344A, H347A), in appropriate transgenic constructs for expression in mammalian cells and *Drosophila* (Fig. S2). The mutations were predicted to destroy the binding of iron to the active site, whilst only minimally disturbing the overall structure of each subunit.

Mutated AOX can be stably expressed in mammalian cells and flies. In order to test its functionality, the expression of the mutated AOX construct (mutAOX) was first verified, following transient transfection into cultured human cells. Based on Western blotting (Fig. 2A), the mutAOX protein was the same size and comparably expressed as wild-type AOX. Next, the mutAOX transgene, under the control of the GAL4-dependent UAS promoter, was introduced into the *Drosophila* genome by targeted insertion at single sites on each chromosome. Parallel control lines were created, containing wild-type AOX and empty vector, inserted at the same sites. Following validation of the insertions by PCR and sequencing, we measured transgene expression directed by the ubiquitous *da-GAL4* driver, at both RNA and protein levels, using QRTPCR (Fig. 2B, C) and Western blotting (Fig. 2D, E).

In both females (Fig. 2B) and males (Fig. 2C), the expression of wild-type and mutAOX were similar at the RNA level, but 3–4 fold less than AOX in the previously created transgenic lines, engineered by random P-element insertion. At the protein level, mutAOX showed slightly lower expression than wild-type AOX in both sexes, and expression was again less than in the previously created lines (Fig. 2D, E).

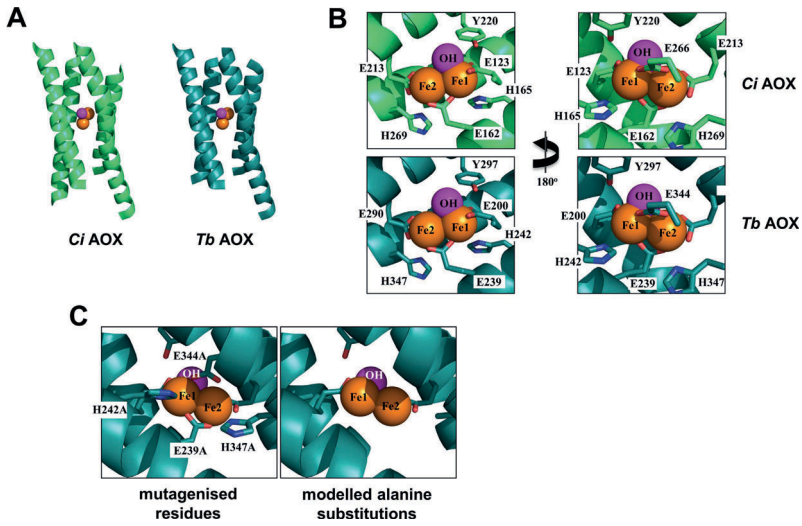

Figure 1. Structural modelling and mutagenesis of active site of *Ciona intestinalis* AOX. (**A**) Model of the active site of the *Ciona* (*Ci*) enzyme, green, compared with the structure of the *Trypanosoma brucei* (*Tb*) AOX, blue. In both cases, the diiron site (iron moieties in orange, hydroxyl in pink) is buried in a four alpha-helix bundle. For clarity, only one protomer is shown. (**B**) Conserved residues binding the diiron centre show an identical arrangement in the *Ci* model (green) as in the *Tb* structure (blue). (**C**) The residues selected for alanine-substitution mutagenesis in the *Ci* enzyme (here shown in blue), alongside the resulting modelled structure.

When expressed ubiquitously using the *da-GAL4* driver, the AOX and mutAOX transgenes produced only very small changes in developmental timing, most of them non-significant compared with the corresponding vector-only line (Fig. 3).

Mutated AOX lacks detectable enzymatic activity. The functionality of the expressed AOX variants was tested by polarography. Permeabilized HEK293T cells, following transient transfection with wild-type AOX, supported approximately 80% of the uninhibited oxygen consumption, in the presence of antimycin. Antimycin-resistant oxygen consumption was undetectable in permeabilized cells transiently transfected with the mutAOX construct or empty vector (Fig. 4A). A similar result was obtained after transfection of *Drosophila* S2 cells. After transfection with either of two different AOX-expressing constructs, whole-cell respiration in the presence of antimycin was 70–73% of the uninhibited rate, but was undetectable in control cells or cells transfected with the mutAOX construct (Table S1). Finally, in homogenates from male transgenic flies carrying targeted insertions at the same locus (on chromosome 2), induced to express the transgene ubiquitously using the *da-GAL4* driver, wild-type AOX supported 14% of the uninhibited substrate oxidation rate in the presence of antimycin (Fig. 4B), whereas mitochondria from mutAOX- or empty vector-transgenic flies showed no antimycin-resistant substrate oxidation. In every polarography experiment, expression of the AOX transgene was verified by Western blotting as per Fig. 2.

Mutated AOX is unable to rescue COX knockdown in flies. The fact that the mutated AOX is devoid of detectable enzymatic activity allowed us to use the newly created transgenic lines to test whether the previously observed phenotypic rescue of flies knocked down for a subunit of cytochrome oxidase (Cox7a) was due to the enzymatic activity of AOX or some other property conferred by the AOX protein, when expressed in *Drosophila*. Moreover, the fact that the newly created transgenic lines express AOX at only about 30% of the level of the lines previously studied, allowed us to test whether phenotypic rescue was quantitatively dependent on AOX expression level. Ubiquitous knockdown of CG9603, the broadly expressed isogene for Cox7a, was previously shown to produce pupal lethality[11], which was rescued by high-level expression of AOX.

To test the new transgenic lines, we first confirmed that the RNAi line used in the experiment was devoid of the additional insertion previously reported to confer pupal lethality unrelated to specific target knockdown[25] (Fig. S3). We then combined the CG9603 RNAi line with AOX and control transgenes, plus the *da-GAL4* driver to induce simultaneous transgene expression and Cox7a knockdown. Wild-type AOX rescued the lethality, as previously (Fig. 5A), whereas mutAOX or the empty vector were unable to do so, confirming that AOX enzymatic activity is required for the rescue.

Next, we investigated the effects of CG9603 knockdown and its potential rescue by AOX, using the neuron-specific driver *elav^{C155}-GAL4*. Previously, it was shown that this produces a locomotor defect in newly

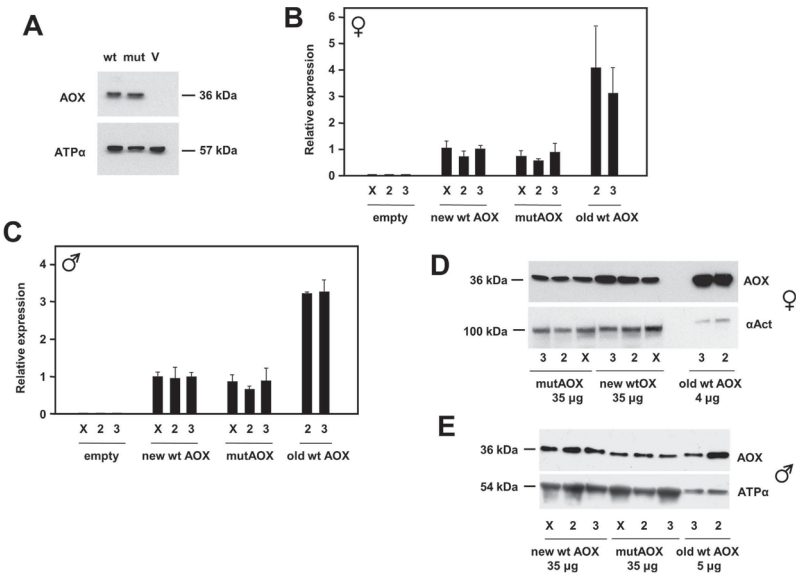

Figure 2. Expression of AOX transgenes in mammalian cells and *Drosophila*. (**A**) Western blot of protein extracts from HEK293T cells transfected with wild-type and mutated AOX constructs (wt, mut) or empty vector (V), probed for AOX and for ATP synthase subunit α as loading control. (**B,C**) Relative AOX expression at RNA level, based on QRTPCR, in (**B**) females and (**C**) males of different *Drosophila* lines transgenic for wild-type or mutated AOX, or empty vector, inserted on chromosomes X, 2 and 3, as shown, in combination with the ubiquitous *da-GAL4* driver. New wt (wild-type) and mutAOX lines were those created by site-specific integration at defined chromosomal sites using the ΦC31 system; old wt AOX lines were UAS-AOX^{F6} (chromosome 2) and UAS-AOX^{F24} (chromosome 3). For males, all values were significantly different from empty-vector lines; old wt AOX lines were significantly different from new wt AOX lines ($p < 0.001$, ANOVA followed by post-hoc Bonferoni-corrected *t* test), but mutAOX and new wt AOX lines were not significantly different from each other. Statistical analysis for females gave similar results, although greater sample-to-sample variation for old wt AOX lines yielded only $p < 0.05$ comparing them with new wt or mutAOX lines. (**D,E**) Western blot of protein extracts from the same flies (amounts as shown), probed for AOX or, as loading control, either ATP synthase subunit α or α-actinin, as indicated.

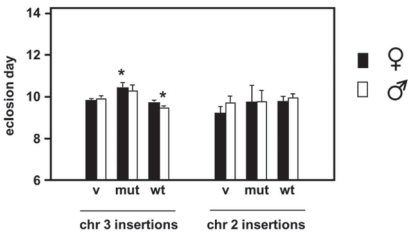

Figure 3. Developmental time to eclosion of AOX transgenic flies. Eclosion day (mean ±SD) for females and males of different *Drosophila* lines transgenic for wild-type (wt) or mutated (mut) AOX, or empty vector (v), inserted on chromosomes X, 2 and 3, as shown, in combination with the ubiquitous *da-GAL4* driver. *denotes significant difference from flies of the same sex from the empty vector line on the same chromosome, $p < 0.05$ (Student's *t* test).

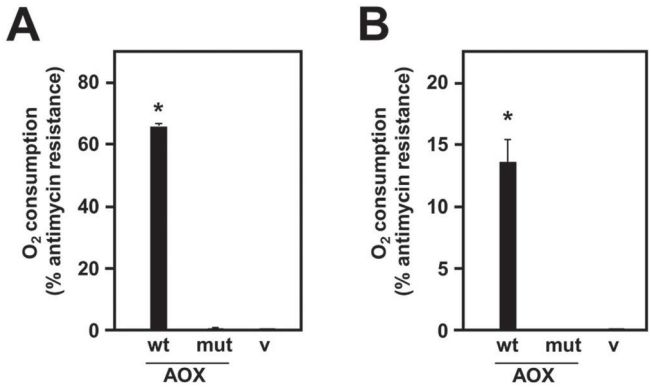

Figure 4. Respirometry of AOX-transfected cells and flies. Oxygen consumption (% resistant to antimycin, as defined in Materials and Methods) of (**A**) permeabilized, transiently transfected cells, and (**B**) homogenates from male transgenic flies induced for expression using *da-GAL4* driver, expressing wild-type (wt) or mutated (mut) AOX or empty vector (v). The flies had transgenic insertions on chromosome 2. *denotes significant difference from vector-only flies.

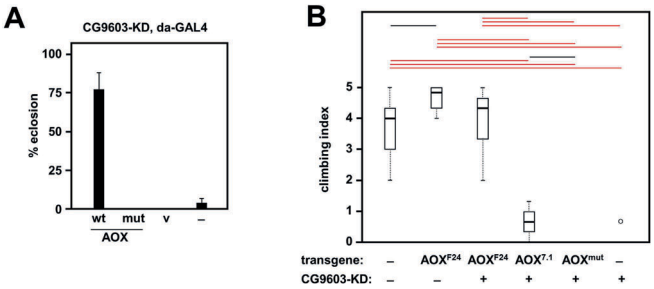

Figure 5. AOX rescue of Cox7a deficiency. (**A**) Survival (%) from egg to eclosion of flies of the indicated genotypes, all bearing the *da-GAL4* driver and the CG-9603 knockdown (RNAi) construct. Lines tested contained either no additional transgene (–), vector only (v), wild-type (wt) or mutated AOX (mut), in each case on chromosome 3. (**B**) Boxplot of climbing index of flies of the indicated genotypes. All flies carried the *elav*^{C155}*-GAL4* driver on chromosome X plus UAS-*Dcr2* with or without the CG9603 knockdown (RNAi) construct on chromosome 2, and the indicated AOX transgene on chromosome 3 (AOX$^{7.1}$ is the ΦC31-targeted insertion). Bars indicate medians, boxes show the first and third quartiles percentiles, whiskers are plotted according to the Tukey scheme (Krzywinski and Altman, 2014). Significant differences based on ANOVA are indicated by horizontal lines (black, red) denoting $p < 0.05$ and 0.001, respectively. A single outlier point is indicated by an open circle.

eclosed flies[11]. To potentiate the phenotype, we included *UAS-Dcr2* in the background, so as to increase the penetrance of RNAi. Without concomitant AOX rescue, the resulting flies showed a severe locomotor defect as measured by their inability to climb the walls of the vial, in a standard negative geotaxis assay (Fig. 5B). High-level expression of AOX produced, as before, a clear rescue, whilst lower-level expression using the newly created transgenic lines produced only a modest phenotypic improvement (wild-type AOX), or no improvement at all (mutAOX, Fig. 5B).

Structural conclusions. Alternative oxidases are members of a superfamily of metalloenzymes, characterized by a common catalytic function of activation of molecular oxygen, and by common structural elements defining the catalytic diiron centre, including the four-helix bundle fold and a motif comprising two histidine residues, four carboxylate groups, and a bridging carboxylate group across the diiron centre[26–28]. The crystal structure of the trypanosomal enzyme indicates that it is a homodimer with each monomer comprising six long and four short α-helices[13]. The subunits interact with each other via α-helices 2, 3 and 4, whereas the hydrophobic region formed by α-helices 1, 2, 4 and 5 is proposed to anchor the protein to the inner surface of the mitochondrial inner membrane. A series of conserved arginine residues, capable of interacting with phospholipid head-groups, may

assist inner membrane anchorage[13]. Our structure modelling of the *C. intestinalis* AOX suggests that the same structural elements are conserved in animal AOXs, and that the enzyme is also a homodimer inserted into the mitochondrial inner membrane.

In addition, the model predicts that the active site, and therefore the mechanism of oxygen activation, are also conserved in animal AOXs. The four-helix bundle, which acts as a structural platform for the binding of the two iron atoms, buries the active site deep in a hydrophobic environment. In *T. brucei* AOX, glutamate residues 123, 162, 213 and 266, in addition to a hydroxo-bridge, are responsible for directly coordinating the diiron centre. The centre is further stabilized by a redox-active tyrosine residue[29,30], Y220, and two histidine residues (H165 and H269), which are within hydrogen-bond distances of E123, E169 and E213. The *C. intestinalis* AOX model indicates that the homologous residues E200, E239, E290, E344, Y297, H242 and H347 organize the active site in the same way.

Functional conclusions. In theory, the mutagenesis of a single glutamate residue should be enough to destabilize the diiron centre[31]. However, taking advantage of the proximity in the DNA sequence of the codons for E239 and H242 and of those for E344 and H347, we were able to create alanine substitutions for four important active site residues simultaneously. According to our model, these mutations should disrupt iron binding, thus generating a mutant devoid of catalytic activity, without any major disturbance to the overall protein structure. These predictions are supported by the fact that the mutant and wild-type proteins were expressed at comparable levels in mammalian cells and in flies, but that no enzymatic activity could be detected.

Importantly, the mutated enzyme was unable to rescue the organismal phenotypes arising from engineered cytochrome oxidase deficiency. In theory, the action of a foreign protein in attenuating such phenotypes could be due to any of several different mechanisms, of which the provision of an enzymatic by-pass for ubiquinol oxidation is only one. In previous work we found that *Ciona* AOX, when expressed in *Drosophila* mitochondria, decreased the net production of mitochondrial ROS even under non-inhibited conditions[10,32]. The mechanism of this remains unknown, but one possibility is that AOX is able to act directly or indirectly as an antioxidant, e.g. by binding and quenching quinone radicals via some other mechanism. Studies in various organisms have supported the idea that a hydrophobic pocket, located between α-helices 2 and 3, binds and channels ubiquinone to the active site[33], which might be involved in such an activity.

A second possibility would be a hormetic response to disruption of the inner mitochondrial membrane or its protein complexes by the foreign protein. The induction of a variety of defence pathways to protect cells from increased ROS, disturbed protein, lipid or redox homeostasis, or altered mitochondrial turnover or dynamics, might equip the organism to cope with the additional but related stresses of respiratory insufficiency. Many studies in model organisms support this concept of 'mitohormesis'[34]. Whilst we cannot rule out that such effects are material in other contexts, our findings do exclude them in regard to the developmental lethality produced by global cytochrome oxidase knockdown, or the locomotor dysfunction resulting from its knockdown specifically in neurons[11]. Based on our findings, that mutAOX cannot compensate these phenotypes, we infer that the rescue of these effects of cytochrome oxidase deficiency by AOX is almost certainly due to its enzymatic activity as a quinol oxidase, though formally we cannot exclude other, unknown effects of iron binding. A requirement for enzymatic activity might not be true of every phenotypic feature conferred by AOX in model organisms. Our findings indicate a robust way to test this in regard to all potential such phenotypes, allowing the mechanisms by which AOX acts to be probed, controlled or verified.

Several quantitative issues are also addressed by our findings. The first is that the extent of phenotypic rescue depends in some instances on the AOX expression level, but in other cases, such as the rescue of the developmental lethality caused by ubiquitous COX knockdown, is an all-or-none phenomenon. We suggest that this reflects a threshold effect wherein even the three-fold lower expression level of AOX, when integrated at specific sites by ΦC31-mediated recombination (in comparison with P element-mediated integrants created previously), exceeds a threshold value required to maintain metabolic homeostasis and complete development. In contrast, the lower expression level of the targeted integrants gave a clearly weaker rescue of locomotor dysfunction, when COX was knocked down only in neurons, roughly in proportion to the decreased expression level.

It may also be noted that the amount of antimycin-resistance conferred upon respiration in homogenates from the targeted integrants was still approximately 14%, compared with approximately 20% for the P element-mediated integrants, even though they are expressed at a much higher level. The level of respiratory antimycin-resistance in the fly may vary between tissues, and this 20% maximum may reflect only the properties of the predominant class of mitochondria. Most of the respiratory capacity in adult flies is vested in the flight muscles, where mitochondria make up almost one-third of the total tissue mass[35]. The apparent upper limit of how much electron flow can be diverted through AOX probably reflects specific features of this tissue and its energetic needs. The limit could be dictated by the constraints of membrane architecture, for example, if much of the ubiquinone pool is channelled directly from complex I to complex III via respiratory supercomplexes, such that it equilibrates only slowly with free ubiquinones available to AOX[36]. Most of the respiratory activity in adult *Drosophila* indeed resides in supercomplexes[37]. Such a phenomenon may account for the inferred threshold effect on the rescue of developmental lethality. Conversely, the organization of the respiratory chain may differ in other tissues, such as in neurons, where a more graded response to the AOX expression level is evident.

In conclusion, mutAOX offers a useful tool for future studies of the mechanism(s) whereby expression of *Ciona* AOX modifies the phenotypes of model organisms, potentially contributing the eventual development of AOX-based therapies.

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Author Contributions

A.A., M.T.O., H.T.J. and P.R. conceived and planned the project. A.A., M.T.O., G.C., C.Y. and P.K.D. conducted the laboratory work and analysis. H.T.J., M.S. and E.D. supervised the laboratory work and contributed analysis and insights. H.T.J. and M.T.O. compiled the figures and drafted the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Diiron centre mutations in *Ciona intestinalis* alternative oxidase abolish enzymatic activity and prevent rescue of cytochrome oxidase deficiency in flies

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SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE

Table S1

Oxygen consumption rate of transfected S2 cells[1]

Treatment	**wt AOX (pAC)**	**wt AOX (pUAST)**	**mutAOX**	**untransfected**
cells only	22.5	17.4	22.6	19.4
+ antimycin	16.3	13.1	1.8	1.9
+ nPG	1.6	1.5	1.8	2.5
% antimycin-resistant[2]	70	73	0	0

[1]$pmol.s^{-1}.ml^{-1}$ from a representative experiment

[2]calculated as 100 times [the oxygen consumption rate in the presence of antimycin – the oxygen consumption rate in the presence of antimycin plus n-PG], divided by [the uninhibited oxygen consumption rate antimycin – the oxygen consumption rate in the presence of antimycin plus n-PG].

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1

Amino acid sequence alignment of eukaryotic mitochondrial alternative oxidase. The selected sequences were retrieved from NCBI Protein database using the sequence of *Ciona intestinalis* AOX as query in BlastP searches. The alignment was performed using the MUSCLE algorithm built in the software MEGA6 with default parameters. Residues highlighted in green form α-helices in the crystal structure of *Trypanosoma brucei* AOX; the ones highlighted in red are responsible for coordinating the binding to the diiron cluster. The last data line indicates invariant amino acids in the taxa shown (*). Note that some of the sequences compiled from genome assemblies are probably incomplete (e.g. *Trichoplax adhaerens*, *Nematostella vectensis*).

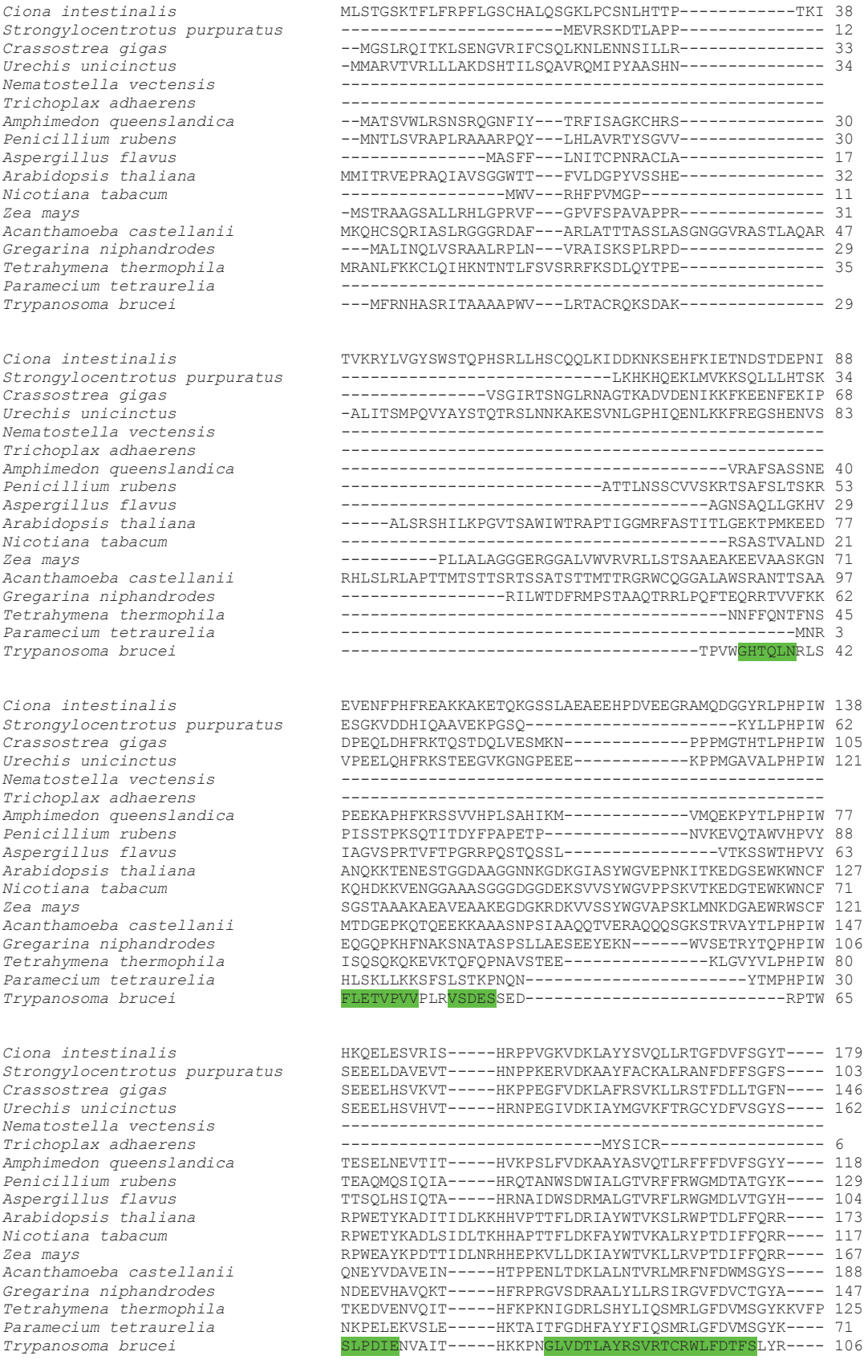
Figure S2

Cloning and mutagenesis strategy. The AOX coding region (red), including its natural stop codon, was amplified from the original vector pMT/V5-His B (abbreviated as pMTb) and recloned into the *Eco*RI site of pUASTattB for site-specific integration into the *Drosophila* genome. Separately, the original plasmid was used for PCR-based *in vitro* mutagenesis (see main text) as indicated (substitutions in green), to create the mutated AOX (mutAOX) coding sequence (green). This was then recloned into ZeroBluntTOPO for use in other systems, and thence into pUASTattB to create the construct for transgenic integration in parallel to the wild-type cDNA.

Figure S3

Verification of transgenic insertion status in VDRC RNAi lines. Green et al (2014) reported

that some lines in the VDRC RNAi collection contain additional insertions, conferring pupal lethality and other phenotypes when activated, due to insertional effects. Insertions at the desired ('good', NA, non-annotated) site can be distinguished from those at the undesired ('bad', A, annotated) site by simple PCR-based analysis specific for the two sites, as recommended by Green et al (2014). In each case, the insertion is distinguished by gel-mobility difference, indicating whether the site is occupied or unoccupied. In the trials shown here, we tested VDRC line 106661 (targeted on CG9603), using as controls the parental line 60100 with insertions at neither site, and line 109338 (targeted on CoIV), which carries insertions at both sites. Line 106661, to be used in the experiments described here, is thus verified to have only the desired insertion, as indicated.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Urechis unicinctus -MMARVTVRLLLAKDSHTILSQAVRQMIPYAASHN---------- 34
Nematostella vectensis ----------
Trichoplax adhaerens ----------
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Penicillium rubens --MNTLSVRAPLRAAARPQY---LHLAVRTYSGVV---------- 30
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Nicotiana tabacum ----------MWV---RHFPVMGP---------- 11
Zea mays -MSTRAAGSALLRHLGPRVF---GPVFSPAVAPPR---------- 31
Acanthamoeba castellanii MKQHCSQRIASLRGGGRDAF---ARLATTTASSLASGNGGVRASTLAQAR 47
Gregarina niphandrodes ---MALINQLVSRAALRPLN---VRAISKSPLRPD---------- 29
Tetrahymena thermophila MRANLFKKCLQIHKNTNTLFSVSRRFKSDLQYTPE---------- 35
Paramecium tetraurelia ----------
Trypanosoma brucei ---MFRNHASRITAAAAPWV---LRTACRQKSDAK---------- 29
Ciona intestinalis TVKRYLVGYSWSTQPHSRLLHSCQQLKIDDKNKSEHFKIETNDSTDEPNI 88
Strongylocentrotus purpuratus ----------LKHKHQEKLMVKKSQLLLHTSK 34
Crassostrea gigas ----------VSGIRTSNGLRNAGTKADVDENIKKFKEENFEKIP 68
Urechis unicinctus -ALITSMPQVYAYSTQTRSLNNKAKESVNLGPHIQENLKKFREGSHENVS 83
Nematostella vectensis ----------
Trichoplax adhaerens ----------
Amphimedon queenslandica ----------VRAFSASSNE 40
Penicillium rubens ----------ATTLNSSCVVSKRTSAFSLTSKR 53
Aspergillus flavus ----------AGNSAQLLGKHV 29
Arabidopsis thaliana -----ALSRSHILKPGVTSAWIWTRAPTIGGMRFASTITLGEKTPMKEED 77
Nicotiana tabacum ----------RSASTVALND 21
Zea mays ----------PLLALAGGGERGGALVWVRVRLLSTSAAEAKEEVAASKGN 71
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Tetrahymena thermophila ----------NNFFQNTFNS 45
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Trypanosoma brucei ----------TPVWGHTQLNRLS 42
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Urechis unicinctus VPEELQHFRKSTEEGVKGNGPEEE----------KPPMGAVALPHPIW 121
Nematostella vectensis ----------
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Amphimedon queenslandica PEEKAPHFKRSSVVHPLSAHIKM----------VMQEKPYTLPHPIW 77
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Zea mays SGSTAAAKAEAVEAAKEGDGKRDKVVSSYWGVAPSKLMNKDGAEWRWSCF 121
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Trypanosoma brucei FLETVPVVPLRVSDESSED----------RPTW 65
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Zea mays RPWEAYKPDTTIDLNRHHEPKVLLDKIAYWTVKLLRVPTDIFFQRR---- 167
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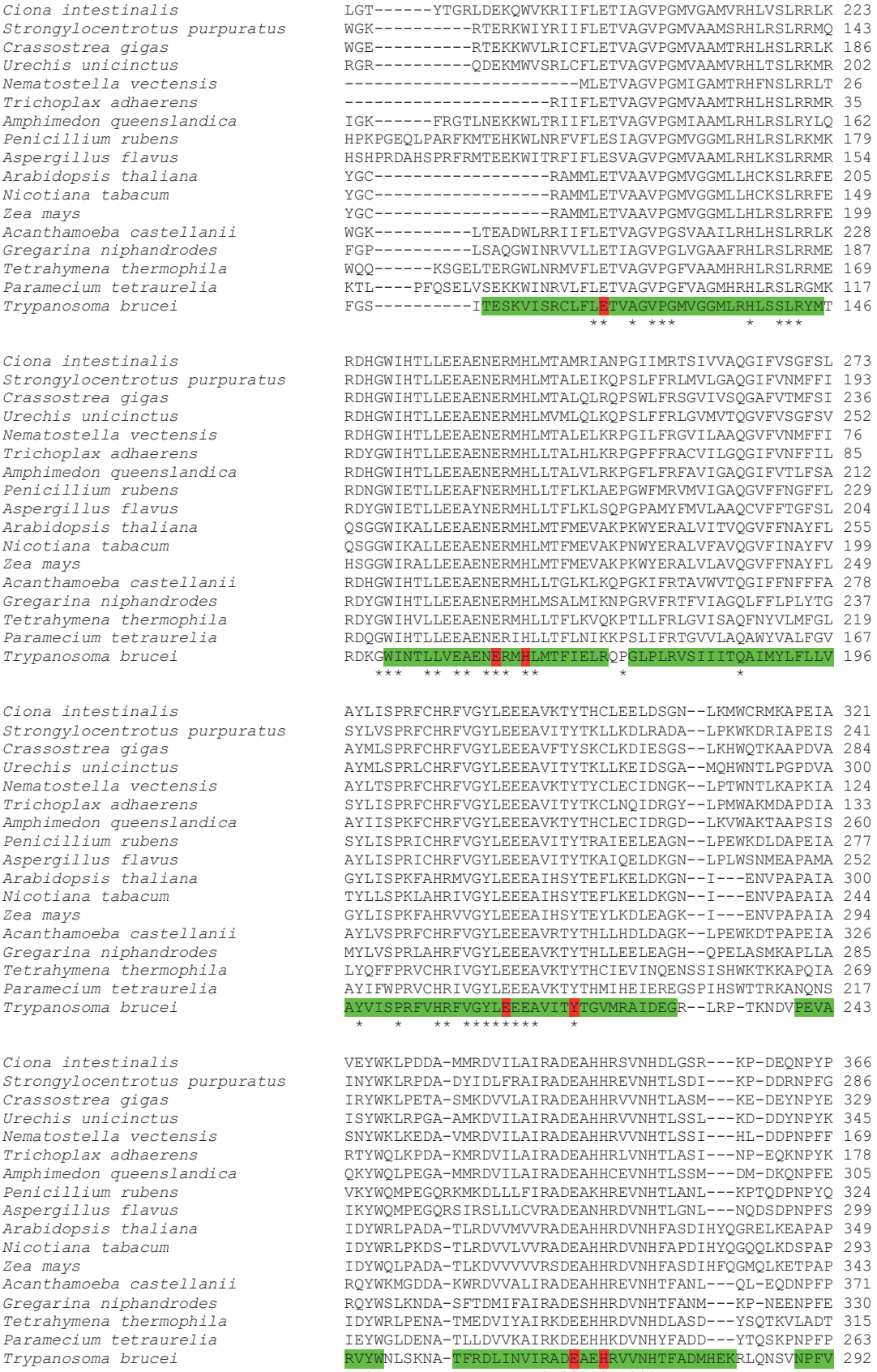
Tetrahymena thermophila TKEDVENVQIT-----HFKPKNIGDRLSHYLIQSMRLGFDVMSGYKKVFP 125
Paramecium tetraurelia NKPELEKVSLE-----HKTAITFGDHFAYYFIQSMRLGFDVMSGYK---- 71
Trypanosoma brucei SLPDIENVAIT-----HKKPNGLVDTLAYRSVRTCRWLFDTFSLYR---- 106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Nicotiana tabacum QSGGWIKALLEEAENERMHLMTFMEVAKPNWYERALVFAVQGVFINAYFV 199
Zea mays HSGGWIRALLEEAENERMHLMTFMEVAKPKWYERALVLAVQGVFFNAYFL 249
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Ciona intestinalis AYLISPRFCHRFVGYLEEEAVKTYTHCLEELDSGN--LKMWCRMKAPEIA 321
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Urechis unicinctus AYMLSPRLCHRFVGYLEEEAVITYTKLLKEIDSGA--MQHWNTLPGPDVA 300
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Zea mays GYLISPKFAHRVVGYLEEEAIHSYTEYLKDLEAGK--I---ENVPAPAIA 294
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Trypanosoma brucei AYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEGR--LRP-TKNDVPEVA 243
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Nematostella vectensis SNYWKLKEDA-VMRDVILAIRADEAHHRVVNHTLSSI---HL-DDPNPFF 169
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Trypanosoma brucei RVYWNLSKNA-TFRDLINVIRADEAEHRVVNHTFADMHEKRLQNSVNPFV 292

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Strongylocentrotus purpuratus     ----------PGE------------------------ 289
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Penicillium rubens                IEYADLSVSHPTKGIDNLRPEGWDRNEIFMGKARTEKS 362
Aspergillus flavus                AKFRNALKE-ASQPLSPVKEHR--------------- 320
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Nicotiana tabacum                 ----------IGYH------------------------ 297
Zea mays                          ----------IEYH------------------------ 347
Acanthamoeba castellanii          ----------PGH------------------------ 374
Gregarina niphandrodes            ----------PGH------------------------ 333
Tetrahymena thermophila           ----------TQDEHYI--------------------- 322
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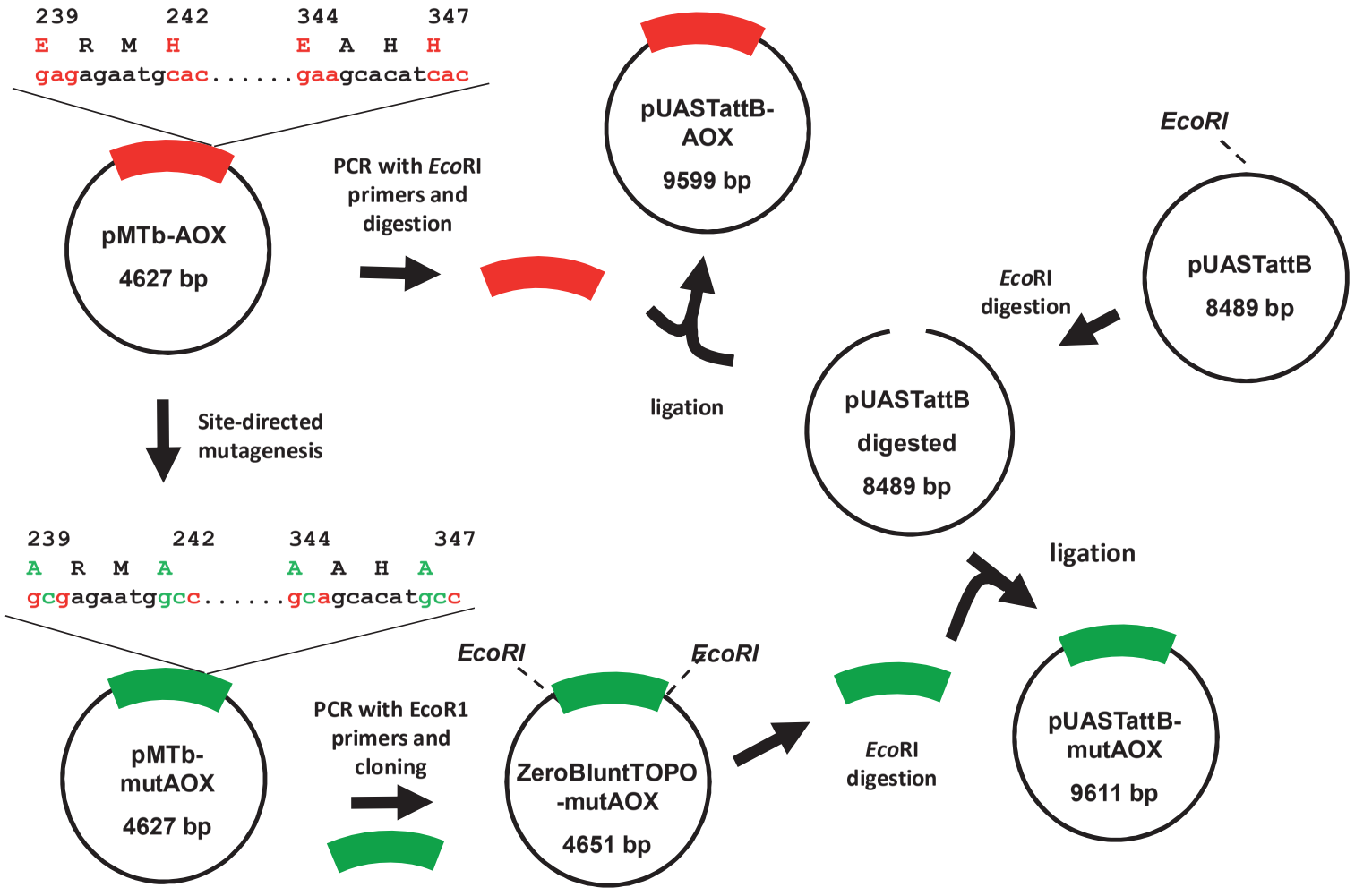
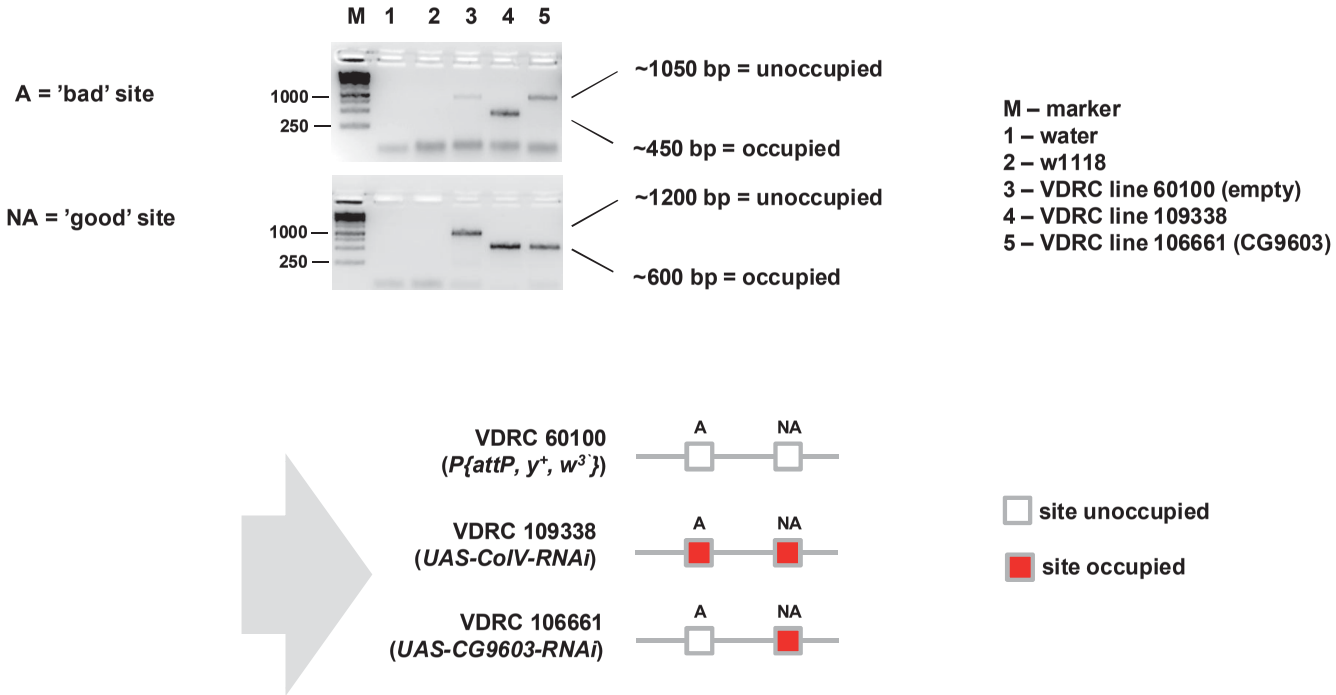


Andjelković et al., Figure S2



Andjelković et al., Figure S3

PUBLICATION II

Ligand-Bound GeneSwitch Causes Developmental Aberrations in *Drosophila* that Are Alleviated by the Alternative Oxidase

Ana Andjelković, Kia K. Kemppainen & Howard T. Jacobs

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Ligand-Bound GeneSwitch Causes Developmental Aberrations in *Drosophila* that Are Alleviated by the Alternative Oxidase

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ABSTRACT Culture of *Drosophila* expressing the steroid-dependent GeneSwitch transcriptional activator under the control of the ubiquitous α-*tubulin* promoter was found to produce extensive pupal lethality, as well as a range of dysmorphic adult phenotypes, in the presence of high concentrations of the inducing drug RU486. Prominent among these was cleft thorax, seen previously in flies bearing mutant alleles of the nuclear receptor Ultraspiracle and many other mutants, as well as notched wings, leg malformations, and bristle abnormalities. Neither the α-*tubulin*-GeneSwitch driver nor the inducing drug on their own produced any of these effects. A second GeneSwitch driver, under the control of the *daughterless* promoter, which gave much lower and more tissue-restricted transgene expression, exhibited only mild bristle abnormalities in the presence of high levels of RU486. Coexpression of the alternative oxidase (AOX) from *Ciona intestinalis* produced a substantial shift in the developmental outcome toward a wild-type phenotype, which was dependent on the AOX expression level. Neither an enzymatically inactivated variant of AOX, nor GFP, or the alternative NADH dehydrogenase Ndi1 from yeast gave any such rescue. Users of the GeneSwitch system should be aware of the potential confounding effects of its application in developmental studies.

KEYWORDS
inducible transgenes
nuclear receptor
Drosophila
cleft thorax
notched wings

The GeneSwitch (GS) system is commonly used to activate transgenes in *Drosophila* in a graded fashion. GS comprises a modified form of the yeast transcriptional activator Gal4, which is covalently linked to the hormone-binding fragment of the progesterone receptor, rendering its transcriptional activity dependent on an exogenously supplied progesterone analog, RU486 or mifepristone (Osterwalder *et al.* 2001). Any transgene governed by the UAS promoter element, rendering it Gal4-responsive, may be induced by the combination of GS and RU486 in a dose-dependent manner. Depending on the promoter to which GS is itself combined, plus its insertion site in the fly genome, drug-inducible transgene expression can be achieved in a wide variety of developmental patterns, cell-types, and overall strengths. Thus, the widely used α-*tubulin-GS* (*tubGS*) and *actin5C-GS* drivers confer ubiquitous, RU486-dependent transgene expression when crossed to lines bearing a UAS-governed transgene. Tissue-specific drivers such as the neuron-specific *elav-GS* enable transgene expression in just one tissue, but again at a level and timing that can be manipulated over a wide range. The use of this system is predicated on the assumption that the expression of GeneSwitch and exposure to RU486 do not themselves produce measurable effects on fly physiology and development, which is supported by controls in many studies.

Our laboratory has made use of this system, for example to express, in *Drosophila*, foreign transgenes coding for nonproton-motive alternative respiratory chain enzymes derived from simpler eukaryotes, such as the alternative oxidase (AOX) from *Ciona intestinalis* (Fernandez-Ayala *et al.* 2009; Kemppainen *et al.* 2014a). When supplied to adult *Drosophila* bearing both *tubGS* and a *UAS-AOX* transgene, RU486 produced dose-dependent transgene expression that saturated at drug concentrations (in fly food) of 100–200 μM (Kemppainen *et al.* 2014a). However, when supplied throughout development, RU486 concentrations two orders of magnitude lower were sufficient to induce maximal expression (Fernandez-Ayala *et al.* 2009). The precise reasons for this discrepancy in required dose are unclear, although early larvae, which are very rapidly growing (Church and Robertson 1966; Watts *et al.* 2006), must absorb larger amounts of drugs added to fly food than

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adults, which do not grow at all and even lose weight during early adult life (Fernandez-Ayala *et al.* 2009).

In this study, we addressed the issue of what happens to development when larvae expressing GeneSwitch drivers (but no other transgene) are exposed to RU486 concentrations in excess of those sufficient to produce maximal transgene expression. We detected a variety of developmental abnormalities dependent on driver expression and drug dose. Surprisingly, expression of AOX, but not other transgenes such as GFP or the yeast alternative NADH dehydrogenase Ndi1, mitigated these effects.

MATERIALS AND METHODS

Drosophila stocks and maintenance

Wild-type (Oregon R), standard transgenic host strains w^{1118} and w^{DAH} (Dahomey) and the UAS-GFP (Stinger) line (insertion on chromosome 2) were obtained from stock centers. The *tubGS* driver line with insertion on chromosome 3 (Sykiotis and Bohmann 2008) was a kind gift from Dr Scott Pletcher (University of Michigan). The *daughterless-GS* (*daGS*) line (Tricoire *et al.* 2009) was a kind gift from Dr Alberto Sanz (Newcastle University, UK). AOX and Ndi1 transgenic flies [lines *UAS-AOX*^{F6}, *UAS-AOX*^{F24}, *tub-AOX*^{7}, *tub-AOX*^{35} *tub-AOX*^{50}, *UAS-AOX*$^{7.1}$ (targeted insertion on chromosome 3) *UAS-AOX*^{mut} (denoted previously as *UAS-AOX*$^{4.1}$, targeted insertion on chromosome 3), and *UAS-Ndi1*^{B20}] were as described previously (Fernandez-Ayala *et al.* 2009; Sanz *et al.* 2010b; Kemppainen *et al.* 2014b; Andjelković *et al.* 2015). Flies were maintained in standard high-sugar medium (Fernandez-Ayala *et al.* 2009) at 25°, on a 12 hr light/dark cycle. Where indicated, medium was supplemented with RU486 (Mifepristone, Sigma) at the concentrations indicated in figures and legends.

Eclosion and phenotypic assays

Crosses were conducted in a minimum of three, usually four to five replicates, as described previously (Toivonen *et al.* 2001; Kemppainen *et al.* 2009). Either the number of flies eclosing or the percentage of pupae that successfully eclosed in individual vials were recorded in different experiments (see figures and legends). The proportion of the eclosed progeny falling into different phenotypic classes was scored by microscopy. Cleft thorax, where subclassified, was scored as mild or severe (heminota clearly separated), with the mildest abnormality, malformed scutellum, scored separately in some experiments. Wing phenotypes were scored as normal or notched, the latter ranging from single notches to grossly malformed wings that in some cases did not inflate properly. Flies showing any of the bristle abnormalities as described below were generally scored as a single category.

Microscopy

Light microscopy images of eclosed adult flies were taken with a Nikon Digital DS-Fi1 High-Definition Color Camera, using the Nikon stereoscopic zoom microscope SMZ 745T run by NIS-Elements D 4.20 software. Fluorescence microscopy of flies used a Zeiss Axio Imager 2 microscope (50 × magnification). Z projection images were generated using Carl Zeiss Zen 2012 software.

Protein analysis by western blotting

Total protein was extracted from batches of 20 pupae crushed in homogenization buffer, and processed as described previously (Andjelković *et al.* 2015). Primary antibodies used were customized rabbit anti-AOX (Fernandez-Ayala *et al.* 2009; 21st Centrury Biochemicals, 1:10,000), and mouse anti-ATP5A (Abcam, 1:100,000), with secondary antibodies as described previously (Andjelković *et al.* 2015).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

tubGS plus high levels of RU486 produce developmental abnormalities

In initial trials, we noticed that doses of RU486 used routinely to induce UAS-dependent transgene expression in *Drosophila*, in combination with the *tubGS* driver in adult flies (200–500 μM; Kemppainen *et al.* 2014a), were lethal when present throughout development. In order to investigate possible mechanisms of this lethality, we reared flies at RU486 doses intermediate between this lethal level, and levels sufficient to induce full dose-dependent transgene expression, which in larvae was only 1–2 μM. In combination with *tubGS*, RU486 at 100 μM was still lethal (Figure 1A), whereas *tubGS* flies reared without drug, or wild-type flies reared at this concentration of RU486, developed normally. At intermediate drug concentrations (5–50 μM, Figure 1, A and B), we observed dose-dependent semilethality, although many of the eclosing flies were very weak and died within 1 d. In addition, even the viable flies displayed a range of dysmorphic phenotypes, illustrated in Figure 2 and Supplemental Material, Figure S1 and File S1, of which the commonest and most striking were cleft thorax (Figure 2, B–D) and notched wings (Figure 2E). The observed phenotypes were of varying severity. For example, some flies had single or multiple notches at the wing margin (Figure 2E), whereas others had wings that failed to inflate (Figure 2F). Cleft thorax ranged from severe, with the heminota completely separated (Figure 2, C and D), to very mild, showing only an abnormal, parted bristle pattern or just a reduced scutellum (Figure 2A). A minority of flies also showed necrotic tissue in the notum area (Figure 2D), leg abnormalities such as overgrown, reduced, and fused leg segments (Figure 2G), externalized trachea (Figure 2H), clefted abdomen (Figure 2I), or a variety of malformations of macrochaetae (supernumerary, missing, kinked, or short bristles, Figure S1). Clefting also extended along the abdomen in some cases (Figure 2I). *tubGS* flies reared without drug, or cultured in RU486 in the absence of *tubGS*, did not exhibit cleft thorax or other developmental abnormalities, indicating that these teratogenic effects require the combination of the modified transcription factor plus the inducing steroid.

We quantified the main classes of abnormality and observed a dose-dependence on RU486 (Figure 3A). Although the proportion of progeny showing the two major dysmorphic phenotypes of cleft thorax or notched wings was already substantial at 10 μM RU486, increasing the dose to 30 μM resulted in a significant increase in the proportion exhibiting cleft thorax, whereas a further increase to 50 μM produced a significantly greater proportion with notched wings.

In order to determine whether the induction of these developmental defects was a general property of GeneSwitch drivers, or a phenomenon specific to *tubGS*, we repeated the experiment using a second GeneSwitch driver under the control of the *daughterless* promoter. In contrast to *tubGS*, *daGS* in combination with 10 μM RU486 produced no clefting and no wing defects. The only developmental abnormality detected was in regard to bristle morphology and organization which, while less frequently observed than with the *tubGS* driver, did show a tendency to rise in frequency as the concentration of RU486 was increased (Figure 3C). However, neither cleft thorax nor notched wings were seen at these elevated drug concentrations, nor even at 100 μM. The difference in the findings between the two drivers is most likely attributable to the level and pattern of expression of the GeneSwitch transcription factor, as reflected in its ability to drive transgene

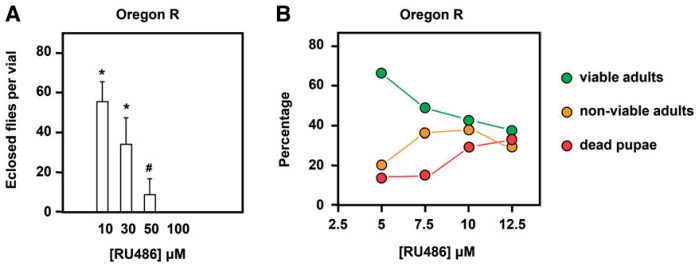

Figure 1 RU486 in combination with *tubGS* produces dose-dependent lethality. (A) Number of *tubGS* progeny eclosing at different doses of RU486 present throughout development, mean ± SD per vial, in OregonR background. Note that at 100 µM, no flies eclosed. * and # indicate significant differences from the next higher concentration tested in pairwise comparisons (Student's *t*-test, $P <$ 0.01 and 0.05, respectively). (B) Proportion (% of pupae formed) of *tubGS* progeny at different doses of RU486 present throughout development; combined data from sets of four vials at a given concentration, set up in parallel, in w^{1118} background. n = 205 (at 5 M), 193 (at 7.5 µM), 210 (at 10 µM), and 146 (at 12.5 µM). *tubGS* plus RU486 produced comparable amounts of pupal lethality also in the CantonS background. SD, standard deviation; *tubGS*; α-*tubulin-GeneSwitch*.

expression, which we profiled quantitatively by western blotting using a *UAS-AOX* reporter (Figure S2A) and spatially using a *UAS-GFP* reporter (Figure S2B). Expression of *UAS-AOX* driven by *daGS* was quantitatively much less than when driven by *tubGS*, even at high RU486 concentrations (Figure S2A). Furthermore, unlike *tubGS*, which was able to drive expression ubiquitously in the developing larva, *daGS* produced transgene expression only in a minority of cells (Figure S2B), including salivary glands, parts of the trachea, some epithelial cells, and segmentally reiterated cell clusters.

Expression of AOX, but not Ndi1 or GFP, rescues cleft thorax caused by tubGS/RU486

We tested whether concomitant expression of other transgenes driven by *tubGS* in the presence of RU486 was able to modify the developmental phenotypes resulting from the driver and drug alone (Figure 4). Once again, neither *tubGS* nor the drug on its own produced cleft thorax (Figure 4A) but, when combined, over 50% of the eclosing progeny manifested severe cleft thorax, and a further 20% showed mild clefting. Coexpression of *Ciona* AOX from either of two *UAS-AOX* transgenic lines (Fernandez-Ayala *et al.* 2009) produced a substantial rescue of the phenotype, with over 50% of the eclosing progeny now showing no cleft, and less than 20% having severe cleft. *UAS-Ndi1* or *UAS-GFP* produced no rescue of the phenotype. Nor did a single copy of AOX, when constitutively expressed under the α-*tubulin* promoter at a much lower level than when driven by *tubGS* (Kemppainen *et al.* 2015). However, five copies of the *tub-AOX* transgene, when present simultaneously, did produce a rescue comparable with that of *UAS-AOX*. Coexpression of *UAS-AOX* with *tubGS* plus drug, in either of two backgrounds commonly used in transgenic studies (w^{1118} and w^{DAH}) also increased the proportion of pupae eclosing (Figure 4B). The simultaneous presence of five *tub-AOX* transgenes (Figure 4C) also substantially rescued the eclosion frequency, as well as the survival of adults immediately after eclosion.

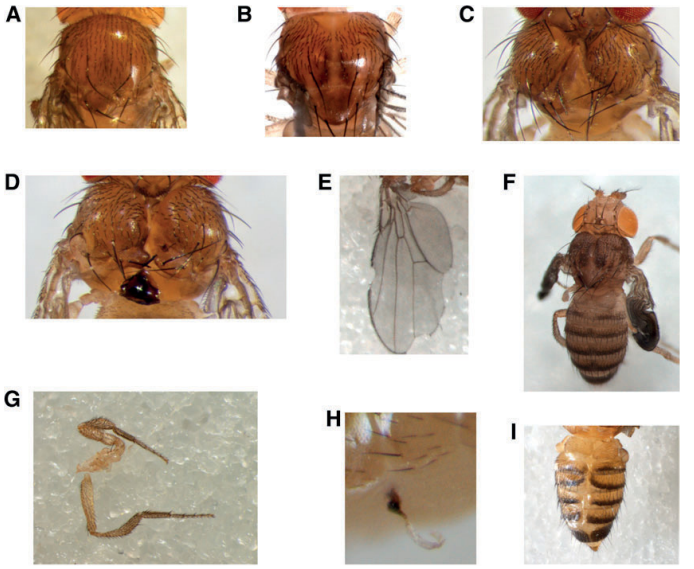

Figure 2 Examples of dysmorphologies produced by the *tubGS* driver in the presence of 10 µM RU486. (A–D) Thoracic abnormalities: (A) missing scutellar part, (B) mild cleft, (C) severe cleft, and (D) necrotic tissue, always localized at the scutellum or notum. (E and F) Wing abnormalities: (E) notched wings, with notches localized on the marginal anterior or posterior side or both, (F) noninflated wings. (G) Leg abnormalities, including overgrown, reduced, and fused leg segments, sometimes present all together. (H) Externalized trachea, always in the ventral abdomen. (I) Abdominal clefting: strong midline splits between all dorsal tergite plates; laterotergites do not fuse at the dorsal midline and remain as hemitergites, with incomplete fusion of abdominal epidermis. These phenotypes were seen in all genetic backgrounds tested (OregonR, CantonS, w^{1118}, and w^{DAH}). *tubGS*; α-*tubulin-GeneSwitch*.

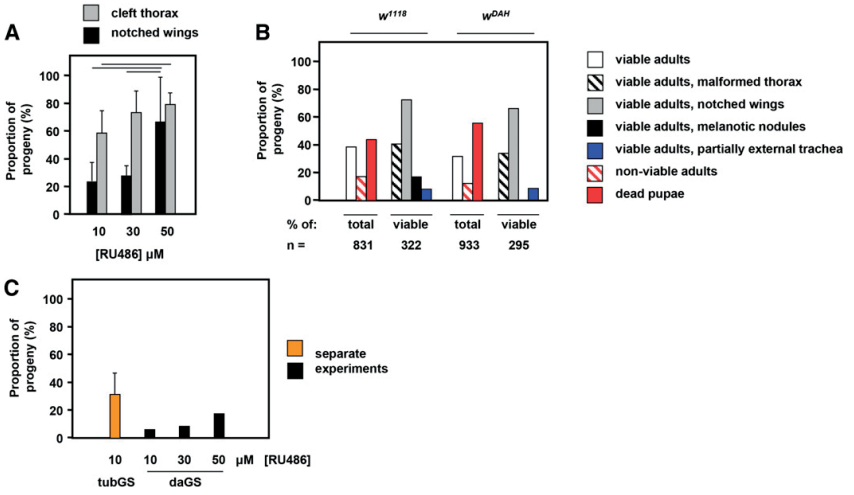

Figure 3 Effects of drug concentration, driver, and genetic background on developmental abnormalities induced by GeneSwitch plus RU486. (A) Proportion of viable adult progeny exhibiting major phenotypic abnormalities as indicated, at different doses of RU486, in the Oregon R genetic background. Mean ± SD for sets of n ≥ 4 independent vials. Horizontal bars denote significant differences for a given phenotypic trait between the stated drug concentrations (Student's *t*-test, $P < 0.05$). (B) Proportion of progeny in different phenotypic classes of *tubGS* flies in the w^{1118} and w^{DAH} backgrounds grown at 10 µM RU486. Note that the adult phenotypes are scored as percentages of the viable adult flies that eclosed. Total numbers of pupae analyzed in each large-scale experiment (n) as indicated. (C) Proportion of adult progeny showing bristle abnormalities, as illustrated in Figure S1, in flies grown at the indicated doses of RU486, bearing the *tubGS* or *daGS* drivers as indicated. Large-scale experiment using the *daGS* driver analyzed $n = 508$ individual adult flies (10 µM), $n = 758$ (30 µM), and $n = 246$ (50 µM). The data for the *tubGS* driver at 10 µM is the mean ± SD for three independent experiments ($n = 89$, 284, and 157 adults analyzed). See also Figure S2. *daGS*, *daughterless-GeneSwitch*; SD, standard deviation; *tubGS*; α-*tubulin-GeneSwitch*.

AOX rescues developmental abnormalities in a dose-dependent manner

We next conducted a large-scale experiment, analyzing almost 2000 individual flies, for each of the major classes of developmental abnormality produced by tubGS in the presence of RU486, in the presence of different UAS-dependent transgenes (Figure 5). As negative control we used strain w^{1118}, the background strain for all the transgenic lines that were crossed in the experiment. To determine whether the failure of a single copy of *tub-AOX* to rescue *tubGS*-induced cleft thorax was due to low expression, we made use of an additional *UAS-AOX* line,

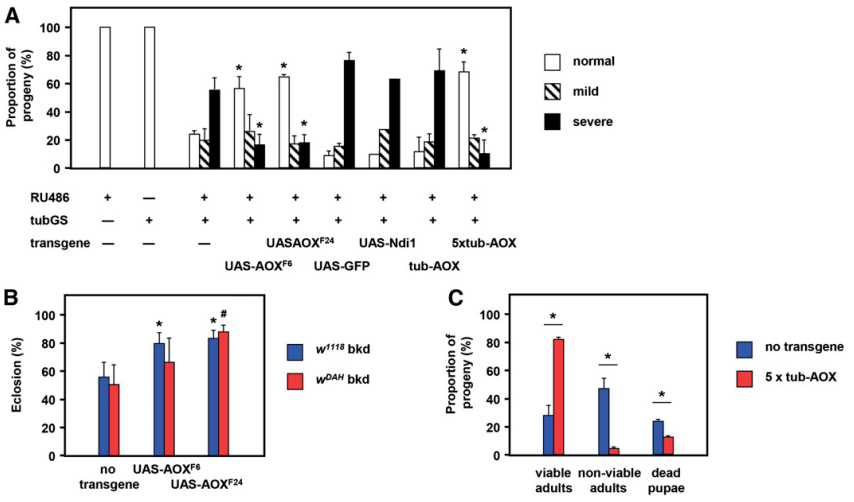

Figure 4 AOX partially rescues cleft thorax and developmental lethality of *tubGS*/RU486. Proportion of adult progeny exhibiting the indicated phenotypes, with hemizygous transgenes as indicated, cultured with (+) or without (–) 10 µM RU486. n ≥ 3 replicate vials for each genotype studied (except *UAS-Ndi*, $n = 2$, hence no error bars shown). Transgenic lines containing *tub-AOX* transgenes (Kemppainen *et al.* 2014) had either a single hemizygous copy or else five copies (two homozygous, plus hemizygous copy on chromosome 3, combined with *tubGS* on the same chromosome). * denotes data classes significantly different from the equivalent class for control lacking any transgene additional to *tubGS* (Student's *t*-test with Bonferroni correction, $P < 0.01$). (B) Proportion of pupae from two different genetic backgrounds (bkd), as shown, eclosing after culture in 10 µM RU486. All pupae carried the tubGS driver and either no other transgene, or either of two different *UAS-AOX* transgenes, as indicated. # and * denote data classes significantly different from nontransgenic flies in the same genetic background (Student's *t*-test, $P < 0.05$ or 0.01, respectively). (C) Proportion of pupae eclosing as viable or nonviable adults after culture in 10 µM RU486. All pupae carried the *tubGS* driver and either no other transgene, or else five copies of tub-AOX transgenes (see above). Nonviable adults were those that died on the day of eclosion. * denotes phenotypic classes of transgenic flies significantly different from corresponding class of nontransgenic flies (Student's *t*-test, $P < 0.01$). AOX, alternative oxidase; GFP, green fluorescent protein; *tubGS*; α-*tubulin-GeneSwitch*.

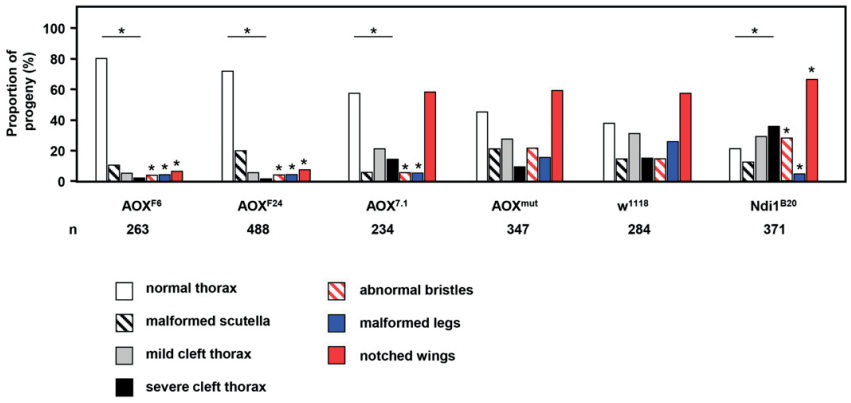

Figure 5 AOX rescues diverse developmental abnormalities produced by *tubGS*. Proportion of progeny hemizygous for both *tubGS* and the indicated transgenes, which exhibited the indicated developmental abnormalities, when reared on food containing 10 μM RU486. The total numbers of flies of each genotype analyzed, in a single large-scale experiment (n), is as shown. See supplemental material for a detailed description of phenotypic categories. Asterisks indicate significant differences ($P < 0.001$) from the w^{1118} background strain hemizygous for *tubGS*, based on chi-squared analysis for each phenotypic category or for the four thoracic phenotypes (normal thorax, malformed scutellum, mild cleft, and severe cleft) considered as a whole. AOX, alternative oxidase; *tubGS*; α-*tubulin-GeneSwitch*.

UAS-AOX$^{7.1}$ (Andjelković *et al.* 2015), showing much lower expression than either of *UAS-AOX^{F6}* or *UAS-AOX^{F24}*. Finally, to confirm that the enzymatic activity of AOX is required for the rescue, we also included a line (*UAS-AOX^{mut}*) expressing a catalytically inactive variant of AOX (Andjelković *et al.* 2015). The proportion of abnormal phenotypes obtained using *UAS-AOX^{mut}* was virtually indistinguishable from the background strain *w^{1118}*, while the weakly expressing *UAS-AOX$^{7.1}$* transgene produced an intermediate spectrum of phenotypes, with cleft thorax, leg, and bristle abnormalities significantly improved over the background strain, but to a much lower extent than seen with the strongly expressing lines *UAS-AOX^{F6}* and *UAS-AOX^{F24}*. *UAS-AOX$^{7.1}$* also produced no rescue of the notched wings phenotype, while *UAS-Ndi1^{B20}* significantly exacerbated all of the abnormal phenotypes compared with the background strain, with the exception of leg malformations, which were decreased in frequency.

DISCUSSION

In this study, we identified a range of developmental abnormalities associated with the use of the *tubGS* driver in combination with RU486. These were seen at concentrations only slightly above those commonly used to induce transgene expression in *Drosophila* during development. At concentrations of 2.5 μM or above, we observed substantial pupal lethality, while at 10 μM or above the majority of viable eclosed adults had visible dysmorphic features, commonly including notched wings and cleft thorax. Importantly, these phenotypes were dependent on both the driver and the drug: neither alone produced any evidence of developmental lethality or abnormality, and the effects did not appear to be background dependent, since they were seen in wild-type OregonR and Canton-S flies, as well as in two white-eyed lines commonly used in transgenic studies. A different GeneSwitch driver, with a much lower and more restricted expression pattern, based on its ability to drive GFP expression (Figure S2), produced only very subtle abnormalities in bristle organization.

Mechanism of developmental disturbance by tubGS/RU486

Previous authors have noted that RU486 treatment alone produces no detectable abnormal phenotypes, although expression of a small number of mRNAs is altered in adults treated with the drug (Etter *et al.* 2005). Given that we also saw no abnormalities from the use of *tubGS* or RU486 on their own, we can exclude the possibility that RU486 binds to or interferes with the activity of known nuclear receptors in *Drosophila* (Fahrbach *et al.* 2012), or that the GeneSwitch transcription factor is able to interact with any of their physiological ligands. However, ligand-bound GeneSwitch may be able to interact either with one or more of these receptors, its targets, or other regulatory factors involved in developmental patterning; for example, by the formation of nonphysiological heterodimers between ligand-bound GeneSwitch and *bona fide* nuclear receptors.

The major dysmorphologies we observed have been reported previously in a variety of mutants, often in combinations similar to those that we observed. Cleft thorax has been reported in mutants of *Ultraspiracle* (Henrich *et al.* 1994), a dimerization partner of the ecdysone receptor and thus one of the key nuclear receptors regulating development progression in the fly. It has also been reported in mutants of the GATA transcription factor *pannier* (Heitzler *et al.* 1996) and the zinc-finger pair-rule transcription factor gene *odd* (Tripura *et al.* 2011). Bristle abnormalities similar to those that we observed are also characteristic of mutants of the dimerization partner of *pannier*, *u-shaped* (Cubadda *et al.* 1997).

Mutants in the components of the AP-1 transcription factor, *jun-related antigen* (homolog of mammalian c-Jun) and *kayak* (homolog of mammalian c-Fos), as well as in the JNK signaling pathway that links AP-1 activity to various upstream developmental signals, cause cleft thorax (reviewed by Zeitlinger and Bohmann 1999; Kockel *et al.* 2001). Defects in JNK signaling also underlie wing defects and leg malformations (Kirchner *et al.* 2007), and have been implicated in midline closure defects in mammals (Chi *et al.* 2005; Zhu *et al.* 2016). Cleft-thorax can result both from downregulation of effectors of JNK signaling, such as the serine protease scarface (Srivastava and Dong 2015), or from mutations in receptor tyrosine kinase Pvr (Garlena *et al.* 2015), an upstream JNK pathway activator (Ishimaru *et al.* 2004; Igaki 2009). Thoracic closure also depends on downstream targets such as proteins implicated in cytokinesis and cell adhesion (Sfregola 2014), as well as intracellular protein trafficking (Thomas *et al.* 2009). Mutants of *blistery*, encoding tensin, result in blistered wings, and interact also with JNK signaling (Lee *et al.* 2003). Overexpression of the inhibitor of matrix metalloproteases (*Timp*) results in pupal lethality and cleft thorax (Srivastava *et al.* 2007). Finally, wing disc-specific knockdown of Tap42, a key regulator of protein phosphatases,

gives rise to cleft thorax and to wing abnormalities similar to some that we observed (Wang *et al.* 2012).

Notched wings are another previously observed phenotype in many mutants, including those affecting the highly pleiotropic intercellular signaling factor Notch (originally discovered by Morgan; Welshons 1958), SNARE-dependent membrane trafficking (Stewart *et al.* 2001), protein phosphatase PP2A (Kunttas-Tatli *et al.* 2009), the RNA-binding fragile X protein FMR1 (Wan *et al.* 2000), and histone deacetylation (Pile *et al.* 2001).

The exact pattern of developmental abnormalities brought about by GeneSwitch together with its ligand appears to reflect the tissue specificity of its expression. Thus, whereas the widely expressed *tubGS* produces a plethora of abnormal phenotypes, *daGS*, with much more restricted larval expression (Figure S2), primarily in segmentally reiterated clusters of cells that might represent larval sense organs (Brewster and Bodmer 1995), has only a single visible phenotype in the adult, affecting the sensory bristles (Figure 3C). The use of other GeneSwitch drivers may help to further clarify how its level and pattern of expression affect the phenotypic outcome.

Finding a common thread through this rather bewildering array of phenotypes and genetic pathways may not be straightforward. However, transcriptional cascades are considered to be the main determinants of developmental processes, and the key system for regulating morphogenesis at pupal stage is the steroid hormone 20-hydroxyecdysone (Riddiford 1993). Thus, an interference with ecdysteroid-dependent transcription is the most parsimonious explanation for the pleiotropic effects we observed, even though molecular details remain to be filled in.

Mechanism of AOX rescue of developmental disturbance by tubGS/RU486

While the observation that GeneSwitch-plus-RU486 can produce a range of developmental abnormalities may be unexpected, their rescue by a mitochondrially localized electron-transfer protein from another phylum is even more surprising. It is important to note that, while the abnormal phenotypes were produced by using an engineered (and thus nonphysiological) transcription factor, and were rescued by a gene from a distant phylum, the effects were systematic in both cases, indicating meaningful underlying biological processes. Thus, the extent of AOX rescue of pupal lethality, cleft thorax, and other dysmorphologies was dependent on the AOX expression level, since strains expressing only at a low level (single-copy of constitutive *tub-AOX*, or low-expressor GAL4-dependent line *UAS-AOX*$^{7.1}$) produced a less dramatic alleviation of the phenotypes studied than the corresponding high-expressors (5 × *tub-AOX*, *UAS-AOXF*^{24}, and *UAS-AOX*^{F6}). Rescue was dependent on the enzymatic activity of AOX and was not seen with an inert reporter protein (GFP) or a different mitochondrially localized electron-transfer protein, yeast Ndi, which appeared to exacerbate some phenotypes. AOX maintains ATP production, redox homeostasis, and metabolic flux under physiological conditions where respiratory complexes III and IV are limiting due to overload, toxins, or genetic damage, and concomitantly limits mitochondrial ROS production consequent upon overreduction of the quinone pool (El-Khoury *et al.* 2014). AOX also has an unexplained antioxidant effect, decreasing net mitochondrial ROS output even under conditions where the respiratory chain is functioning normally (Fernandez-Ayala *et al.* 2009; Sanz *et al.* 2010a).

How this links to a global alleviation of developmental perturbations brought about by interference with transcriptional cascades or cell signaling is far from clear. In a general sense, our findings hint at a common metabolic regulation of transcription, such as evidenced previously by AMPK sirtuins or PARP (Kraus and Lis 2003; Ghosh *et al.* 2010; Gut and Verdin 2013; Schiewer and Knudsen 2014; Salminen *et al.* 2016), although none of these is obviously implicated, so a novel pathway may be involved. In mice, nuclear receptors are responsive to a variety of metabolic effectors, which can also be microbiome-dependent (Montagner *et al.* 2016), while cross-talk between nutrient-based sensors and nuclear receptors is dependent on mitochondrial stress signals and influences mitochondrial gene expression (Kang *et al.* 2015).

Many transcription factors, including nuclear receptors such as LXRα (Serviddio *et al.* 2013) or NR4A1 (Shimizu *et al.* 2015) in mammals, are known to be activated in response to oxidative stress (Lavrovsky *et al.* 2000), and redox regulation of nuclear receptors such as the glucocorticoid receptor (Tanaka *et al.* 1999) is well established. AOX may therefore act by providing a general dampening of ROS, normalizing developmental outcomes dependent on such receptors, with which GeneSwitch plus RU486 interferes. An exhaustive study using different ROS scavengers may shed further light on this.

Another possibility is based on the observation that synthesis of 20-hydroxyecdysone requires mitochondrial Fe-S cluster-containing proteins dependent on frataxin (Palandri *et al.* 2015) and mitoferrin (Llorens *et al.* 2015). Because Fe-S proteins are highly susceptible to ROS damage, a general ROS dampening effect of AOX may counteract transcriptional interference from ligand-bound GeneSwitch, simply by boosting endogenous ecdysteroid synthesis.

Recommendations on use of GeneSwitch drivers

The GeneSwitch system was originally elaborated using other drivers than *tubGS*, *i.e.*, those linked to the neuron- and muscle-specific *elav* and *Mhc* promoters, respectively (Osterwalder *et al.* 2001), or for specific expression in other tissues such as the fat body (Roman *et al.* 2001). Subsequently, the "ubiquitous" GS drivers (such as *tubGS* and *Actin5C-GS*) have been brought into use for inducing broad expression, both in adults and larvae (Ford *et al.* 2007; Waskar *et al.* 2009; Wigby *et al.* 2011; Paik *et al.* 2012; Kuo *et al.* 2012; Kemppainen *et al.* 2014a,b; Sun *et al.* 2014; Da-Rè *et al.* 2014).

Our work raises at least two concerns. First, the visible interference with developmental processes at saturating or near-saturating drug concentrations, using the *tubGS* driver, indicates the need for rigorous controls and cautious interpretation of all data obtained using this driver during development. Furthermore, we obviously cannot rule out subtler but also biologically significant effects that did not have visible manifestations, even at lower drug concentrations than those employed here. Second, other GeneSwitch drivers activated during development may also be vulnerable to such effects, since our data indicate that they depend on the drug and the transcription factor in combination, which applies wherever they are colocated. An example would be the recently published use of a GeneSwitch driver to overexpress malic enzyme (Kim *et al.* 2015). The driver in this example was originally reported to induce expression in the adult abdominal fat body (Hwangbo *et al.* 2004), although Kim *et al.* (2015) found that expression in larvae was instead driven in the salivary glands, Malpighian tubule, and part of the gut. In this particular paper, the appropriate controls without the transgene were indeed implemented for the adult (see Supplementary Table 1C of Kim *et al.* 2015), but some questions remain. The driver plus drug alone did not affect the body weight of L3 larvae (Figure 3A of Kim *et al.* 2015), but effects on stress resistance and lifespan in such controls were not documented. The concentrations of RU486 used by Kim *et al.* (2015), *i.e.*, 2.5–10 μg/ml, corresponding with 5.8–23 μM, were within the range in which we saw major developmental effects using the *tubGS* driver. Similarly, in flies expressing GeneSwitch in specific endocrine cells during

development, using a customized driver and RU486 at even higher concentrations from larval L2 stage onwards (Cho *et al.* 2014), clear developmental abnormalities were attributed to knockdown of a nuclear receptor, although driver-plus-drug controls were not included in all of the experiments reported. Some phenotypes observed (Figure 3 of Cho *et al.* 2014) resemble those that we report here (pupal lethality, uninflated wings, abdominal clefting, and leg malformations). While their interpretation that these are due to disrupted ecdysone signaling may be correct, an effect of GeneSwitch plus RU486 in the target cells cannot be excluded. Phenotypic rescue by injected ETH (Table 2 of Cho *et al.* 2014) confirmed the involvement of disrupted ecdysis, but not the underlying causes thereof. A further possible example already reported in the literature is the effect of the abdominal fat body-specific GeneSwitch driver on lifespan, when RU486-containing food was supplied in the adult to drive the supposedly inert GFP transgene (Ren and Hughes, 2014. RU486-dependent lethality in larvae containing the Elav-GeneSwitch driver (Shen *et al.* 2009), and embryonic lethality produced by either the Elav- or Actin5C-GeneSwitch drivers plus maternal RU486 (Landis *et al.* 2015), have been previously reported.

We would recommend that future users of all GeneSwitch drivers should routinely include otherwise nontransgenic controls bearing the drivers, plus and minus drug, in all experiments. Based on our findings (Figure S2B), the *daGS* driver is clearly not ubiquitous, despite the fact that the *daughterless* gene itself, as well as the "standard" *daGAL4* drivers, do show widespread expression.

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Ligand-bound GeneSwitch causes developmental aberrations in *Drosophila* that are alleviated by the alternative oxidase

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SUPPLEMENTARY DATA

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1

Examples of sensory bristle defects on the notum, produced by GeneSwitch drivers in presence of RU486

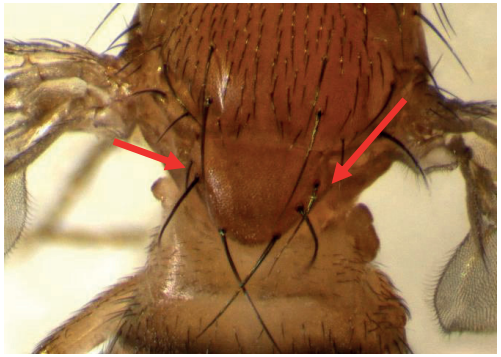
Wild type flies have four scutellar bristles at the notum. (A) Extra (ectopic, six) scutellar macrochaetes. (B) Missing (three) scutellar macrochaetes. (C) One of the scutellar macrochaetes is shorter and missoriented (red arrow); one of the notum macrochaetes appears bent (white arrow). (D) Bent scutellar macrochaete.

Figure S2

Profiling of expression driven by the *tubGS* and *daGS* drivers

(A) Western blot of protein extracts from pupae expressing AOX (line *UAS-AOX*^{F6}), driven by the indicated drivers and RU486 concentrations, and probed for AOX and for ATPα as loading control. (B) Apotomized fluorescent micrographs of L3 larvae expressing nucleus-localized GFP (line *UAS-GFP* Stinger), driven by the indicated drivers in presence of 10 μM RU48. Scale bars 0.5 mm. In other images, *daGS* clearly produced low-level expression of GFP also in parts of the trachea and some epithelial cells as well as the salivary glands and segmentally reiterated cell clusters most obvious in the image shown.

A



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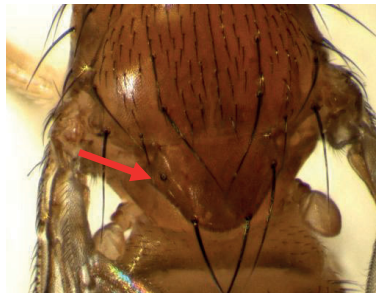
B



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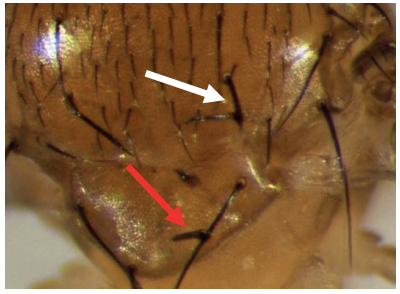

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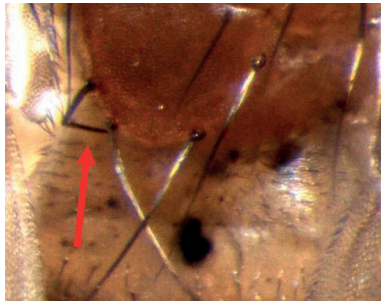

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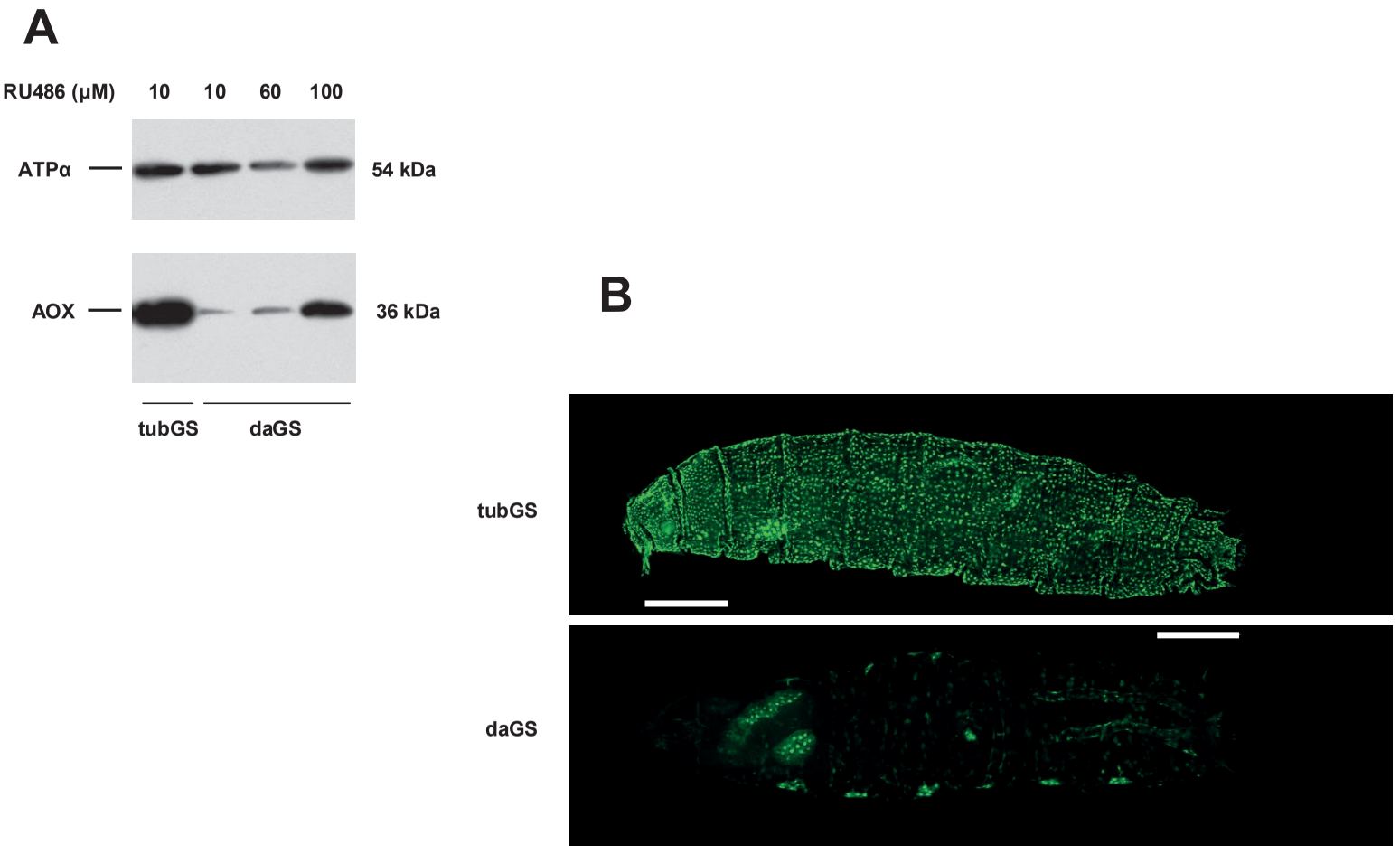

Andjelković et al, Figure S2

PUBLICATION III

Expression of the Alternative Oxidase Influences Jun N-Terminal Kinase Signaling and Cell Migration

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RESEARCH ARTICLE

Expression of the Alternative Oxidase Influences Jun N-Terminal Kinase Signaling and Cell Migration

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ABSTRACT Downregulation of Jun N-terminal kinase (JNK) signaling inhibits cell migration in diverse model systems. In *Drosophila* pupal development, attenuated JNK signaling in the thoracic dorsal epithelium leads to defective midline closure, resulting in cleft thorax. Here we report that concomitant expression of the *Ciona intestinalis* alternative oxidase (AOX) was able to compensate for JNK pathway downregulation, substantially correcting the cleft thorax phenotype. AOX expression also promoted wound-healing behavior and single-cell migration in immortalized mouse embryonic fibroblasts (iMEFs), counteracting the effect of JNK pathway inhibition. However, AOX was not able to rescue developmental phenotypes resulting from knockdown of the AP-1 transcription factor, the canonical target of JNK, nor its targets and had no effect on AP-1-dependent transcription. The migration of AOX-expressing iMEFs in the wound-healing assay was differentially stimulated by antimycin A, which redirects respiratory electron flow through AOX, altering the balance between mitochondrial ATP and heat production. Since other treatments affecting mitochondrial ATP did not stimulate wound healing, we propose increased mitochondrial heat production as the most likely primary mechanism of action of AOX in promoting cell migration in these various contexts.

KEYWORDS AP-1, Jun N-terminal kinase, alternative oxidase, transcription, wound healing

Cell migration is an essential process in animal development, as well as in tissue repair. It has been widely studied in model systems, where the focus has been largely on mechanosensation and mechanotransduction (1, 2). The transcriptional and cytoskeletal regulation of cell migration ensures coordination and an ability to respond to extrinsic and intrinsic cues (2). At the cellular level, the most studied mammalian model is the scratch or wound-healing assay, in which a linear scratch is made in a confluent monolayer of cells, which then migrate to close the gap at a measurable rate (3). In *Drosophila* development, cell migration has been studied in embryogenesis, in the process of dorsal closure (4, 5), and later on during metamorphosis, when many of the same genes are involved in thoracic closure (6). This process involves cells everting from the wing imaginal discs, which spread over the preexisting larval epidermis (7). These migrating cell sheets eventually fuse at the midline to create a closed epithelial layer that gives rise to the cuticular structures of the dorsal thorax.

In an earlier study (8), we reported that the process of dorsal thoracic closure is disrupted by the expression of a commonly used, inducible driver of transgene expression, GeneSwitch, in the presence of the inducing steroid RU486. GeneSwitch is a modified version of the *Saccharomyces cerevisiae* transcription factor GAL4 incorporating the ligand-binding domain of the progesterone receptor so as to place it under

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steroid control (9, 10). Since progesterone or its analogues are not found in *Drosophila*, it had been assumed that GeneSwitch plus RU486 would be phenotypically inert in otherwise wild-type flies, which is indeed the case in adults. Although cleft thorax was the most dramatic and frequent phenotype observed in GeneSwitch-expressing flies reared throughout development on RU486-containing medium, other developmental dysmorphologies were also observed, including wings with apoptotic regions, abnormal or missing bristles, and cleft abdomen.

In the course of these studies, we observed that coexpression of the mitochondrial alternative oxidase (AOX) from *Ciona intestinalis* was able to revert the cleft thorax and other dysmorphological phenotypes brought about by GeneSwitch plus RU486 (8). Expression of an otherwise inert transgene, such as green fluorescent protein (GFP), the alternative NADH dehydrogenase Ndi1 from yeast, or even a catalytically inactive variant of AOX, was unable to correct GeneSwitch-plus-RU486-induced cleft thorax (8).

AOX represents an accessory component of the mitochondrial respiratory chain (RC), which is found in microbes, plants, and some metazoan phyla but not insects or vertebrates (11). AOX provides a non-proton-motive bypass for complexes III (cIII) and IV (cIV) of the standard RC. In various contexts, it is able to relieve metabolically deleterious stresses arising from damage, toxic inhibition, or overload of the RC (11, 12). Furthermore, when expressed in human cells, flies, or mice, *Ciona* AOX can alleviate the damaging phenotypes associated with RC inhibition (13–19). However, the link between respiratory homeostasis and dysmorphologies resulting from GeneSwitch plus RU486 is unknown.

These findings prompted us to test whether AOX could revert the cleft thorax phenotype brought about by genetic manipulations in the signaling network that maintains the migratory behavior of the cell sheets everting from the wing discs. Three such classes of mutants have been studied. First, cleft thorax is manifested by specific, recessive alleles of the gene encoding the *Drosophila* RXR homologue, ultraspiracle (usp), which acts as a dimerization partner for the ecdysone receptor (20). Second, compound heterozygotes for another essential transcription factor, the GATA factor pannier (pnr), also give rise to this phenotype (21). One *pnr* allele used in these studies is pnr^{MD237}, a hypomorph created by insertion of GAL4 into the promoter region for one of the two antagonistic pnr isoforms. This allele was originally isolated in an enhancer-trap screen and has proven useful as a driver of transgene expression in the specific domain of *pnr* expression in the dorsal epithelium; thus, it is often referred to as *pnr*-GAL4.

Third, cleft thorax results from mutations in the Jun N-terminal kinase (JNK) signaling pathway (4) (Fig. 1A). JNK (22) is a member of the mitogen-activated protein (MAP) kinase family that activates the AP-1 transcription factor by phosphorylating its c-Jun subunit (23). AP-1 has a plethora of cellular roles, which include the regulation of cell migration both in development (24) and in pathology, e.g., tumor invasion (25). It is also subject to many types of regulation (26). JNK is itself activated by a variety of stresses through a classic kinase cascade (27, 28). In the context of thoracic closure, the initiating stimulus appears to be the engagement of receptor tyrosine kinase pvr (29) (PDGF [platelet-derived growth factor] and VEGF [vascular endothelial growth factor receptor] receptor related). Cleft thorax is produced by mutant alleles of the JNK kinase (JNKK) *hemipterous* (*hep*) (30) or of the AP-1 subunit *kayak* (*kay*; the *Drosophila* ortholog of mammalian c-*Fos*) (31). The use of *pnr*-GAL4 or other drivers to bring about the local downregulation of JNK targets, such as *scarface* (serine protease) (32), or overexpression of the AP-1 target *puckered* (*puc*; a phosphatase regulator of JNK via a negative feedback loop) (33) or the *tissue inhibitor of metalloproteases* (*Timp*) (34) can also produce cleft thorax, while downregulation of *puc* can rescue cleft thorax caused by mutations of *hep* (30). One key target of JNK in dorsal closure (35, 36) is the transforming growth factor β family member decapentaplegic (dpp). In thoracic closure, *dpp* promotes the migration of cells at the imaginal leading edge (7), but it acts in a parallel pathway rather than

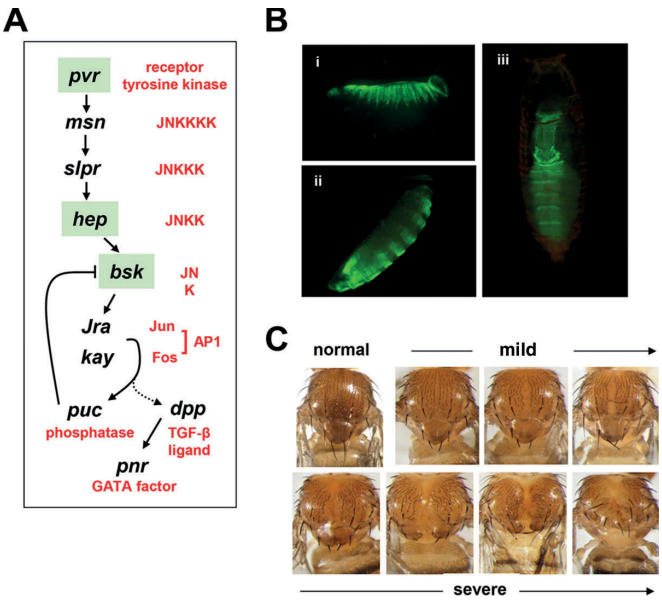

FIG 1 Cleft thorax produced by downregulation of JNK signaling. (A) Summary of the main steps in the JNK signaling cascade in *Drosophila* thoracic development indicating *Drosophila* genes by their standard symbols and their functional assignments in red text. The dotted line to *dpp* represents its activation by AP-1 in embryonic dorsal closure but not in pupal thoracic closure. *pnr* is activated by *dpp* to regulate the dorsal phenotype. The steps indicated with a green background are the ones that were clearly influenced by AOX, based on the data presented later in the paper. TGF-β, transforming growth factor β. (B) Live-cell imaging of a 13- to 15-h-old embryo (i), an L3-stage larva (ii), and a pupa (iii) of flies expressing GFP under the control of the *pnr*-GAL4 driver (original pnr^{MD237} strain). (C) Examples of thoracic phenotypes scored as normal, mild, or severe, with arrows indicating the trend within each class toward more severe cleft thorax phenotypes.

downstream of JNK (30). One key target of *dpp* in thoracic closure is *pnr* (37). A *dpp* homologue in mammals is similarly involved in palatal closure (38).

We therefore set out to test whether AOX could rescue cleft thorax when induced by manipulations of the JNK pathway and the associated gene network described above.

RESULTS

AOX expression mitigates cleft thorax due to downregulation of JNK signaling. We first confirmed, using RNA interference (RNAi) and the *pnr*-GAL4 (pnr^{MD237}) driver, that the downregulation of key components of the JNK signaling cascade (Fig. 1A) (22) in the mediodorsal region during *Drosophila* development resulted in a phenotype of cleft thorax. After verifying the expression pattern conferred by *pnr*-GAL4, using the GFP reporter already present in the pnr^{MD237} stock (Fig. 1B; see Table S1 in the supplemental material), we combined it with RNAi insertions targeted against *basket* (*bsk*; encoding JNK), *hemipterous* (*hep*; encoding JNKK), *misshapen* (*msn*; JNKKKK), and *PDGF- and VEGF-receptor related* (*pvr*; encoding the receptor tyrosine kinase at the top of the cascade [Table S2]). These all produced a cleft thorax phenotype of various severities (see Fig. 1C for examples), according to the tested construct/insertion and temperature. The two isolates of the *pnr*-GAL4 driver gave indistinguishable morphological phenotypes and were therefore used interchangeably in the remainder of the study. Under conditions producing the clearest phenotypes, but avoiding substantial lethality (except in the case of *pvr*, where it was unavoidable), we then combined these with expression constructs for AOX or for a control transgene, the GFP gene (Fig. 2 and 3).

For *bsk* and *hep* we tested multiple RNAi lines (Table S1), each producing cleft thorax when combined with the *pnr*-GAL4 driver. Although the severity of cleft thorax varied

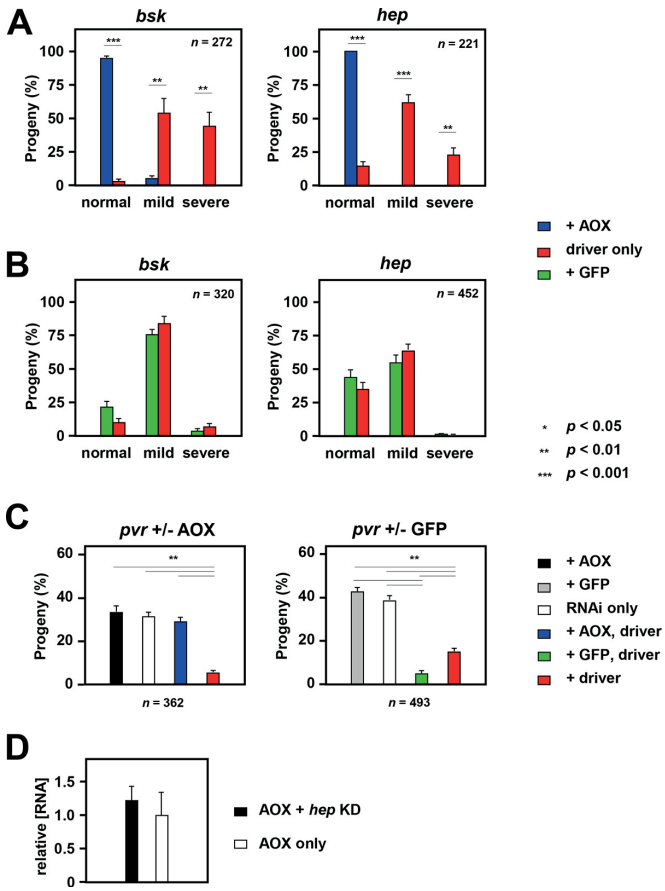

FIG 2 AOX rescues cleft thorax produced by downregulation of JNK signaling. (A, B) Effects of coexpressing AOX (A) or GFP (B) on the proportion of different phenotypic classes resulting from knockdown of *bsk* and *hep*, using the *pnr*-GAL4 driver and RNAi lines KK 104569 (*bsk*) and GD 47507 (*hep*). For details of the crosses, see Table S2 in the supplemental material. The data represent the means ± SEM for nine replicate vials in each experiment, with *n* indicating the total number of flies analyzed in each case. Statistically significant differences between the proportions of AOX- or GFP-expressing and -nonexpressing flies of different phenotypic classes are shown. *P* values, as indicated, were determined by paired, two-tailed Student's *t* test with Bonferroni correction. (C) Effect of coexpressing AOX or GFP on pupal semilethality caused by knockdown of *pvr* (RNAi line KK 105353) using the *pnr*-GAL4 driver. For details of the crosses, see Table S2 in the supplemental material. The data represent the means ± SEM for nine replicate vials in each experiment, with *n* indicating the total number of flies analyzed in each case. Statistically significant differences between classes are indicated, with *P* values being determined by analysis of variance with the Tukey *post hoc* honestly significant difference (HSD) test. Note that conversion to percentages for each vial corrects for differential lethality and for other vial-specific anomalies. (D) qRT-PCR analysis of AOX RNA (means ± SD; $n = 3$) in hemizygous *UAS-AOX*^{F6} transgenic flies that were also hemizygous for *pnr*^{MD237} (*pnr*-GAL4), with or without the *hep* RNAi construct of line GD 47509. Values were normalized against those for RpL32 and then against the mean value for flies expressing AOX only, to generate the relative values shown. KD, knockdown.

slightly between experiments, expression of AOX (Fig. 2A and 3A and B) but not that of GFP (Fig. 2B) led to a significant and substantial shift toward a wild-type phenotype in the progeny of *bsk* or *hep* knockdown flies. Two different RNAi lines for each gene showed the same effect (Fig. 3E to G). Any contribution to the alleviation of the phenotype from promoter dilution was excluded by measuring the amount of AOX RNA driven by *pnr*-GAL4 in pupae with and without one of the double-stranded RNA (dsRNA) constructs for *hep*, which showed no significant difference (Fig. 2D).

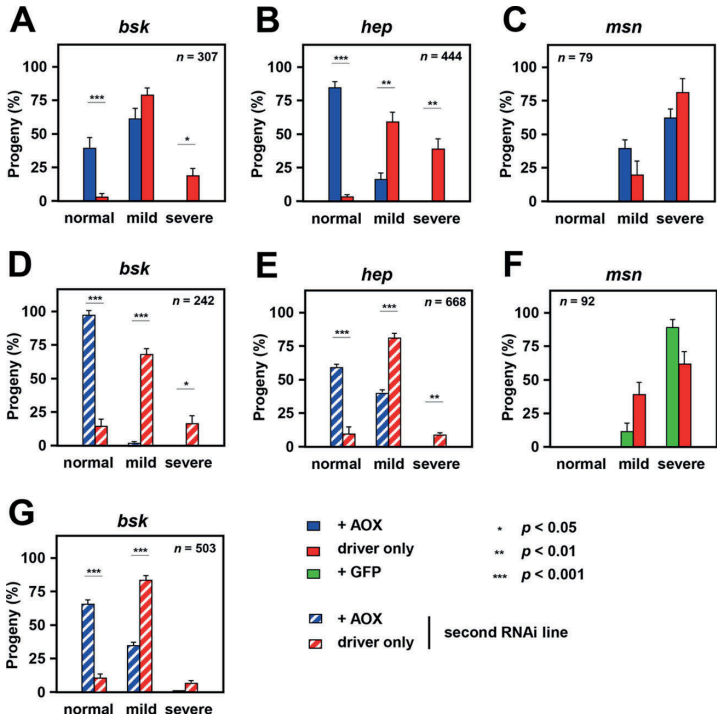

FIG 3 Confirmation of AOX rescue of cleft thorax caused by JNK knockdown. Effects of coexpressing AOX or GFP on the proportion of different phenotypic classes resulting from knockdown of *bsk*, *hep*, and *msn*, using the *pnr*-GAL4 driver. (A, B) Repeats of experiments whose results are shown in Fig. 2A. (C) Results of assays with RNAi line KK 101517 (*msn*) with coexpression of AOX. (D, E, G) Repeats of the experiments whose results are shown in Fig. 2A, using alternate RNAi lines, GD 38138 (*bsk*) and GD 47509 (*hep*). (F) Results of assays with RNAi line KK 101517 (*msn*) with coexpression of GFP. For details of the crosses, see Table S2 in the supplemental material. The data represent the means ± SEM for nine replicate vials in each experiment, with *n* indicating the total number of flies analyzed in each case. Statistically significant differences between the proportions of AOX- or GFP-expressing and -nonexpressing flies of different phenotypic classes are shown. *P* values, as indicated, were determined by paired, two-tailed Student's *t* test with Bonferroni correction. Note that conversion to percentages for each vial corrects for differential lethality and for other vial-specific anomalies.

For *msn* knockdown, very few flies eclosed using the available RNAi line, and AOX or GFP expression produced no significant change in phenotype, despite a trend toward the wild type for AOX (Fig. 3C) and increased severity in the case of GFP (Fig. 3F). A *pvr* knockdown line was pupal semilethal when combined with the *pnr*-GAL4 driver, even at 18°C. As a result, the number of eclosing progeny was insufficient to enable a statistically meaningful analysis of the thoracic phenotype according to severity, but the mean proportion of progeny with cleft thorax was about 80% in this and parallel *pvr* knockdown experiments. Coexpression of AOX, but not GFP, gave substantial rescue of semilethality (Fig. 2C), with 71% (75/105) of the eclosing flies having a normal thorax.

AOX expression can influence mammalian cell migration. The failure of thoracic dorsal closure during *Drosophila* development indicates a defect in cell migration, which AOX expression was able to correct. To test the generality of this finding, we conducted cell migration assays in mammalian cells. Mouse embryonic fibroblasts (MEFs) were isolated from AOX hemizygous mice and wild-type littermates and immortalized using a standard retroviral transduction procedure with viruses encoding human papillomavirus 16 (HPV16) oncoproteins E6 and E7 (39). AOX-endowed immortalized MEFs (iMEFs) showed an increased speed of wound closure in the standard

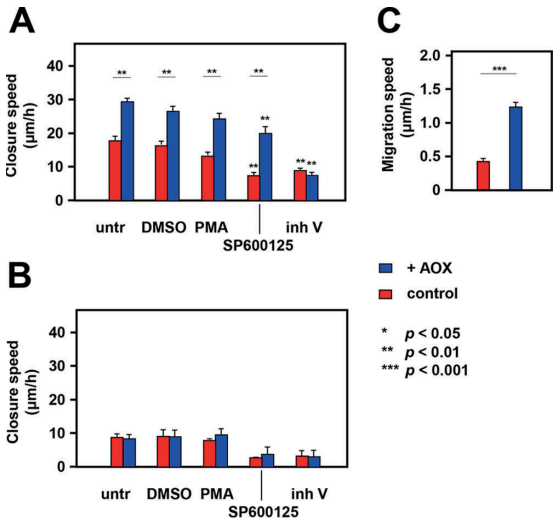

FIG 4 Effects of AOX on mammalian cell migration. (A, B) Rate of wound closure in scratch assay of cultured wild-type iMEFs (control) and AOX hemizygous iMEFs (A) and primary MEFs (B) at passage 6, as indicated, either untreated (untr), treated with only 0.2% DMSO, with PMA (20 mM), with SP600125 (20 μM in 0.2% DMSO), or with JNK inhibitor V (inh V; 20 μM), as shown. Asterisks above the bars indicate a statistically significant difference (determined by one-way analysis of variance with the Tukey *post hoc* HSD test) from untreated cells of the given genotype. Asterisks joining the bars indicate statistically significant differences between the genotypes for a given treatment, based on the same statistical analysis. For clarity, other significant differences are not shown. All data points are based on three biological replicates, each analyzed in triplicate, except for DMSO only, which used only two biological replicates. For the primary MEFs in panel B, the means ± SD are for pooled data from two cell lines of each genotype analyzed in triplicate at passage 6. (C) Rate of migration of single iMEFs of the indicated genotypes. Asterisks denote statistical significance, as shown (Student's *t* test, unpaired; $n = 31$ for control iMEFs; $n = 21$ for AOX-endowed iMEFs).

scratch assay (Fig. 4A), which was maintained in the presence of various drugs, notably, phorbol myristate acetate (PMA), an indirect activator of AP-1-dependent transcription (acting via protein kinase C), and the JNK inhibitor SP600125. However, JNK inhibitor V decreased the rate of wound closure of AOX-endowed iMEFs to the same level as wild-type iMEFs. The scratch assay conducted on primary MEFs (at passage 6) revealed no difference in migration rate between AOX-endowed and control MEFs (Fig. 4B). All primary lines migrated much more slowly than iMEFs, with inhibitor V also producing substantial cell death. The rate of single-cell migration of AOX-endowed iMEFs was also significantly greater than that of control iMEFs (Fig. 4C).

AOX expression has no systematic effect on c-Jun phosphorylation. We next tested the same set of JNK modulators for their effects on c-Jun phosphorylation at JNK target sites (40) Ser63 and Ser73, which has been shown to promote wound healing in the scratch assay (41). This was done in iMEFs (Fig. 5A), as well as in two other cell lines, the HEK293-derived AP-1 transcriptional reporter line used later in the study (HEK-AP1) (Fig. 5B) and human fibroblast line BJ-5ta (Fig. 5C). Only SP600125 decreased the amount of phosphorylated c-Jun, whereas JNK inhibitor V instead increased it, as did PMA. The presence of AOX did not influence c-Jun phosphorylation at these sites in iMEFs (Fig. 5A), although it did appear to potentiate the effect of inhibitor V in an AOX-expressing BJ-5ta cell clone (Fig. 5C).

AOX does not rescue cleft thorax caused by manipulation of AP-1 expression or other targets. We reasoned that directly downregulating c-Jun or its dimerization partner, c-Fos, encoded in *Drosophila* by *Jun-related antigen* (*Jra*) and *kayak* (*kay*), respectively, should produce effects that largely override its regulation by JNK and the beneficial effects of AOX. Accordingly, coexpression of AOX had only a slight effect on the severity of cleft thorax induced by knockdown of *kay* or *Jra* using the *pnr*-GAL4

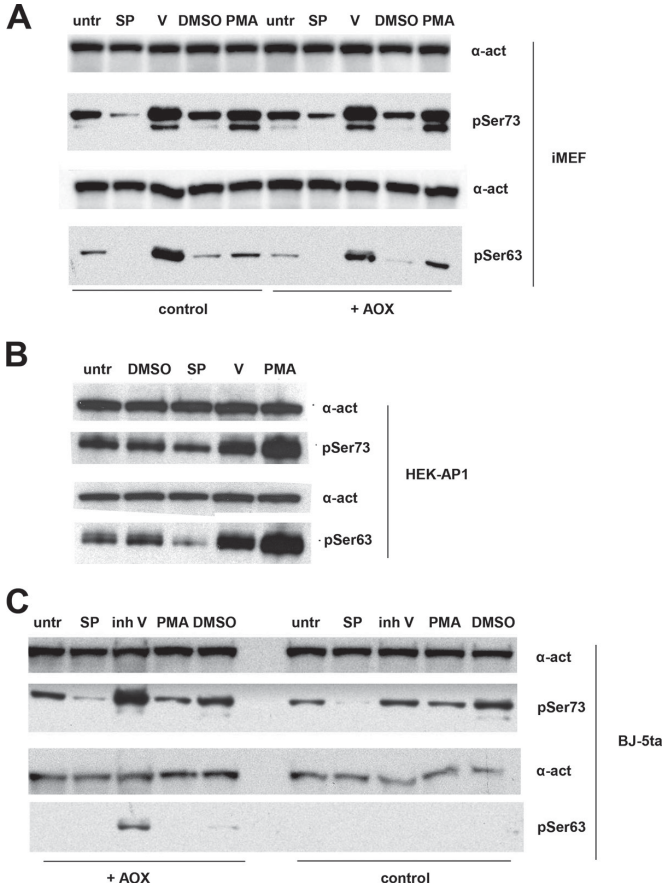

FIG 5 Phosphorylation status of JNK target residues in c-Jun. Western blots of whole-cell protein extracts from control and AOX-expressing iMEFs (A), HEK-AP1 cells (B), and AOX-expressing or control human BJ-5ta fibroblasts (C) untreated (untr) or treated with JNK modulators, as shown: 0.2% DMSO, 20 μM SP600125 (SP) in 0.2% DMSO, 20 μM JNK inhibitor V (V or inh V), or 8 nM PMA. The molecular weights of the major bands detected by each antibody, inferred from size markers run on all gels, were as expected (100 kDa for α-actinin [α-act], 47 kDa for c-Jun phosphorylated at residue Ser73 [pSer73] or Ser63 [pSer63]). Separate blots were initially probed for pSer73 or pSer63, and then in both cases the blots were reprobed for α-actinin as a loading control. Drug concentrations were based either on dose-response curves obtained using the HEK-AP1 cell transcriptional reporter system (for PMA and JNK inhibitor V; see Table S3 in the supplemental material) or on trials to determine the highest concentration at which there was no evidence of substantial cell death (for SP600125). Blot images were optimized for brightness and contrast, rotated, and cropped with the addition of white frames or dividers for clarity, but with no other manipulations.

driver (Fig. 6A and B). Similarly, AOX was unable to rescue the lethality caused by overexpression of the AP-1 target, *puc*, which also antagonizes the action of *bsk* (Fig. 6C). Note, however, that this result may be trivial, since *puc* overexpression generates a severe embryonic phenotype, due to the inhibition of dorsal closure.

As discussed earlier, *pnr* is considered to act in thoracic closure via a pathway parallel to the JNK pathway. Since the *pnr*-GAL4 line pnr^{MD237} is also a *pnr* hypomorph, we combined it with the pnr^{D1} mutant as a compound heterozygote, producing, as expected, a phenotype of severe cleft thorax (Fig. 6D). This was not alleviated by AOX, whether it was supplied using a constitutive or a GAL4-dependent transgene (Fig. 6D). Our findings are consistent with the inference that AOX acts on JNK signaling upstream of AP-1 but cannot compensate for a deficiency of AP-1 itself nor of a parallel pathway also required for thoracic closure.

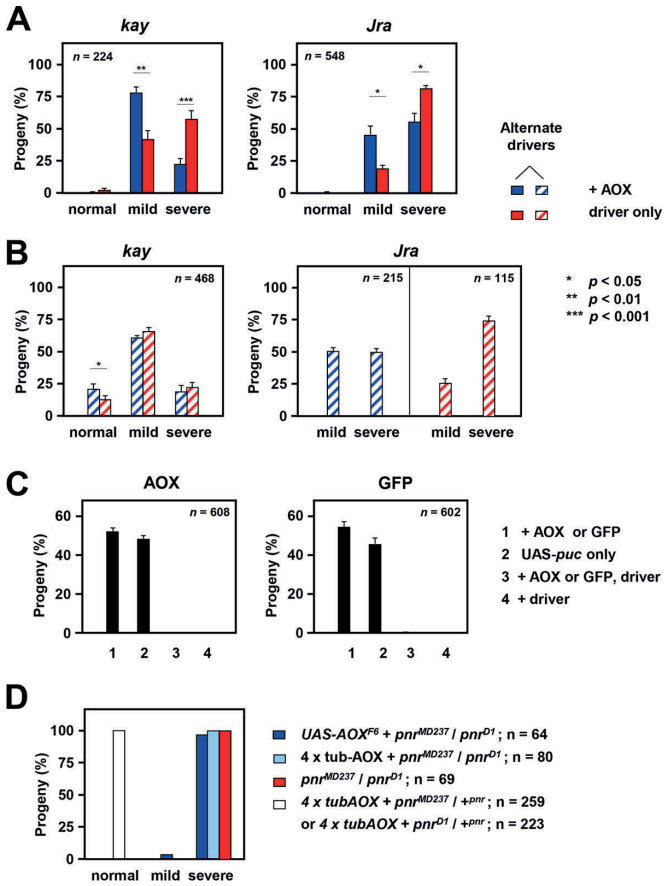

FIG 6 AOX does not rescue cleft thorax produced by altered expression of AP-1 or other targets. (A, B) Effects of coexpressing AOX on the proportion of different phenotypic classes resulting from knockdown of *kay* (at 25°C) and *Jra* (at 18°C), using the *pnr*-GAL4 driver and RNAi lines GD 6212 (*kay*) and KK 107997 (*Jra*) (A) and alternate RNAi lines GD 19512 (*kay*) and GD 10835 (*Jra*) (B). For details of the crosses, see Table S2 in the supplemental material. Because the GD 10835 (*Jra*) construct is carried on chromosome X, two parallel crosses were required to test the effects of AOX expression in each sex, and statistical analysis was not meaningful in this case. The data represent the means ± SEM for nine replicate vials in each experiment, with *n* indicating the total number of flies analyzed in each case. Statistically significant differences between the proportions of AOX-expressing and -nonexpressing flies of different phenotypic classes are shown. *P* values, as indicated, were determined by paired, two-tailed Student's *t* test with Bonferroni correction. Note that *Jra* knockdown using RNAi line KK 107997 (*Jra*) was lethal at 25°C and that AOX did not rescue this lethality. (C) Effect of coexpressing AOX or GFP on pupal lethality caused by overexpression of *puc* under the control of the *pnr*-GAL4 driver. Progeny classes are as indicated, and all contained, in addition, the UAS-*puc* overexpression construct. For details of the crosses, see Table S2 in the supplemental material. The data represent the means ± SEM for nine replicate vials in each experiment, with *n* indicating the total number of flies analyzed in each case. Note that conversion to percentages for each vial corrects for differential lethality and other vial-specific anomalies. (D) Phenotypes of pnr^{MD237}/pnr^{D1} compound heterozygotes with and without the presence of AOX transgenes, as indicated. Neither the GAL4-driven *UAS-AOX*^{F6} transgene nor homozygosity for the *tub-AOX* transgenes on chromosomes 2 and X produced the rescue of the strong cleft thorax phenotype. Note that because progeny phenotypes were essentially uniform for a given genotype, no meaningful variances could be calculated.

AOX does not influence AP1-dependent transcription in cultured cells. To investigate the mechanism by which AOX impacts the outcome of JNK signaling, we tested whether it influences transcription directed by AP-1. Using a well-established luciferase-based AP-1 reporter system (42) and a variety of different expression con-

structs for AOX, we tested whether AOX expression in *Drosophila* S2 cells was able to alter AP-1-dependent transcription under different conditions of JNK pathway activation. First, we compared the transcriptional readout in cells cotransfected with the reporter plasmids and with AOX cloned into the copper-inducible expression vector pMT/V5-His B, with its natural stop codon, with that in cells transfected with the empty vector. JNK pathway activation was achieved using the pUAST-Hep^{act} plasmid, included in all transfections in combination with pAct-Gal4, which promotes pUAST-Hep^{act} transcription by constitutive expression of Gal4. Transfection efficiency was controlled by the inclusion of a constitutively expressed plasmid encoding renilla luciferase, which can be experimentally distinguished from the firefly luciferase of the reporter construct. Finally, to measure background transcription independently of AP-1, the system includes a mutated version of the reporter (which was used as an alternative in transfections), to which AP-1 does not bind.

Despite the complexity of this system, it gave clear-cut results. AOX produced no significant change in AP-1-dependent luciferase expression under both basal and JNK-activated conditions (Fig. 7A; Table S4). Next, we tested reporter cells cotransfected with a plasmid (pAC/AOX) (43) directing constitutive AOX expression under the control of a β-actin promoter versus cells cotransfected with the empty vector. Again, AOX expression had no effect on the transcriptional readout (Fig. 7B; Table S4). Using a system in which JNK pathway activation and AOX induction were brought about simultaneously by expression of the exogenous transcription factor Gal4, but this time using a control plasmid harboring a catalytically inactive, mutated AOX, we again found no effect of AOX (Fig. 7C: see also the results of a parallel experiment in Table S4). AOX also produced no significant difference in AP-1-dependent transcription in cells where *hep* had been knocked down (Fig. 7D; Table S4).

We conducted a similar exercise in mammalian cells, using an HEK293 cell-derived reporter cell line (here designated HEK-AP1), stably transduced with lentiviral constructs expressing AOX or, as a control, the mutated, catalytically inactive variant (mutAOX). Successful transduction and cell cloning at limiting dilution were verified via the fluorescence conferred by the cotransduced marker GFP, and AOX functionality was verified by respirometry (Table S5). Although individual HEK-AP1 cell-derived clones showed a variable degree of AP-1-dependent transcriptional activity, AOX-expressing and control cell clones showed a similar susceptibility to the effects of the JNK antagonists SP600125 and inhibitor V (Fig. 7E). Surprisingly, SP600125 increased rather than decreased the transcriptional readout, despite the fact that it inhibited c-Jun phosphorylation (Fig. 5B), although it did modestly suppress PMA-activated transcription in the reporter line (Fig. 7E).

Antimycin A differentially stimulates the migration of AOX-expressing cells. To gain insight into the intracellular process(es) underlying the enhanced migratory behavior of AOX-expressing cells, we tested the effects of sublethal doses of various metabolic effectors on the relative rates of migration of AOX-expressing versus control iMEFs. In an initial experiment (Fig. 8A), we tested various oxidative phosphorylation inhibitors, antioxidants, and protease inhibitors in the wound-healing assay for a differential effect on AOX-expressing cells. For further study, we selected three treatments that appeared to give a differential effect (antimycin A, oligomycin, and mitoquinone mesylate [MitoQ]), together with two that did not (rotenone and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone [FCCP]), and measured wound closure in four independent experiments. Antimycin A had a significantly different effect on the migration of AOX-expressing MEFs versus wild-type MEFs (Fig. 8B), stimulating the migration of the former but suppressing that of the latter, whereas MitoQ, rotenone, oligomycin, and FCCP had no significant effects. To understand the implications of these findings for the mechanism by which AOX promotes cell migration, we checked the effects of AOX expression on respiration in the cell lines tested (Fig. 8C). AOX had no significant effect on whole-cell respiration or on permeabilized cell respiration on cI-, cII-, and cIV-linked substrates. However, in the presence of antimycin A, it enabled

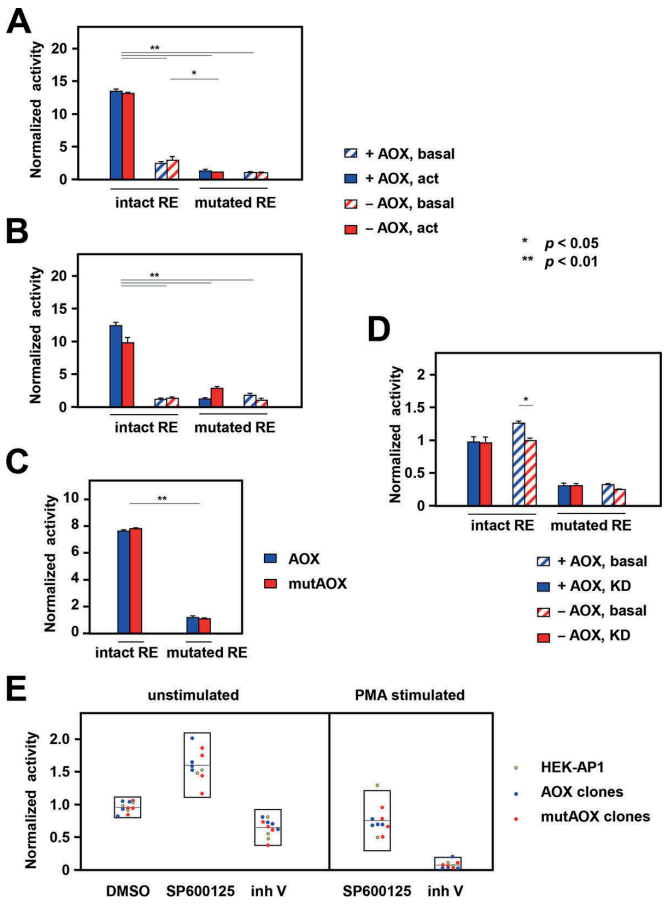

FIG 7 Effects of AOX on AP-1-dependent transcription. (A to D) Firefly luciferase activity in extracts of S2 cells cotransfected with pMT-AOX (or empty vector) (A), pAC/AOX (or empty vector) (B), or pUASTattB-AOX (or pUASTattB-mutAOX) (C), with pUAST-Hep^{act} together with pAct-Gal4 (or not) to activate it (A to C) or following 5 days of dsRNA treatment (knockdown) for *hep* (D), and with reporter constructs containing intact (TRE-fluc) or mutated (mRE-fluc) phorbol ester-response elements (RE), as indicated, and pAct-RL for normalization. All data were first normalized for transfection efficiency, based on renilla luciferase activity, followed by renormalization against basal activity for the relevant control cells, i.e., the empty vector (A, B, D) or pUASTattB-mutAOX (C). The results for AOX-expressing and AOX-nonexpressing cells in any category were not significantly different from each other (Student's *t* test, two-tailed, unpaired), apart from one case in panel D, as indicated. Statistically significant differences between data classes were determined by one-way analysis of variance with the Tukey *post hoc* HSD test; for clarity, only comparisons between the pairs which were coherent are shown, except in panel D, where some significance values differed, as shown. See also Table S4 in the supplemental material for the results of repeat and parallel experiments. act, activated. (E) Firefly luciferase activity in extracts of HEK-AP1 reporter cells and clones derived from them transduced with AOX- or mutAOX-expressing lentiviral constructs (see Table S5 for clone characterization) and treated with the indicated drugs: 0.2% DMSO, 20 μM SP600125 in 0.2% DMSO, or 20 μM JNK inhibitor V. Data were normalized against the values for the corresponding untreated cells (unstimulated) or for the corresponding cells stimulated with 8 nM PMA, as shown. Box plots indicate the means and the 95% confidence intervals for each data set.

almost 80% of the uninhibited respiration rate in permeabilized cells, driven by succinate. This capacity actually exceeded the measured rate of whole-cell respiration under uninhibited conditions, implying that the capacity for AOX-mediated respiration was sufficient to maintain normal respiratory electron flow in iMEFs in the presence of antimycin A.

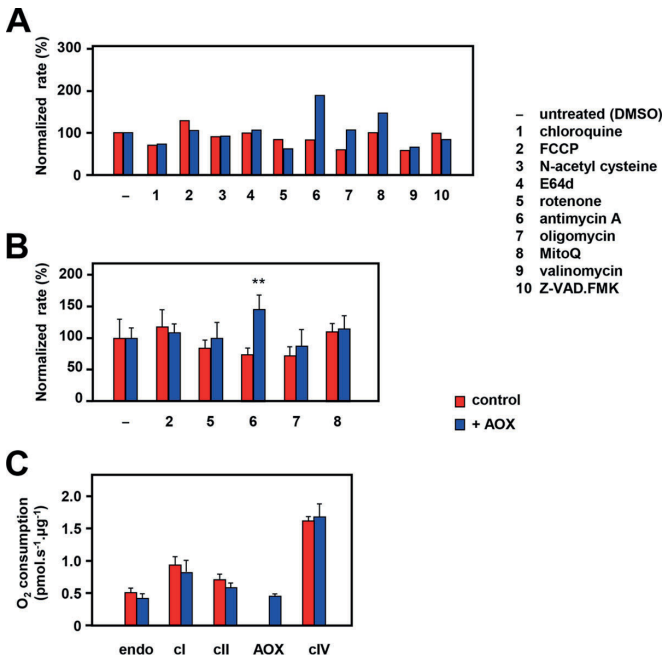

FIG 8 Modulation of iMEF migration by metabolic inhibitors. (A, B) Relative rate of wound closure for wild-type iMEFs (control) and AOX-hemizygous iMEFs treated either with DMSO only (—) or with the indicated drugs (see Materials and Methods). Data are normalized against the closure rate for the given cell line treated only with DMSO. (A) Preliminary experiment with 9 different drugs. (B) For the 5 drugs indicated, the data represent the means ± SD from 4 independent experiments. Asterisks indicate statistically significant differences (Student's *t* test, $P < 0.01$) between drug-treated and untreated (DMSO only) cells of the given genotype. (C) Respiratory oxygen consumption of control and AOX-expressing iMEFs is indicated as follows: endo, endogenous whole-cell respiration; cI, cII, cIV, and AOX, respiration of permeabilized cells driven by cI-, cII-, or cIV-linked substrate mixes or by cII-linked substrate mix in the presence of antimycin A (denoted AOX), respectively.

DISCUSSION

In this study, we showed that the expression of AOX is able to promote cell migratory behavior in two different models: in *Drosophila*, AOX expression corrected thoracic closure defects produced by impaired signaling at several steps in the JNK pathway (summarized in Fig. 1A), while AOX-expressing iMEFs (but not primary MEFs) showed enhanced migration, which was abolished by JNK inhibitor V. In contrast, AOX was unable to correct cell migration defects resulting from downregulation of the main JNK substrate, the c-Jun subunit of AP-1, or by manipulation of other factors, such as the AP-1 downstream target *puc* or the *pnr* transcription factor. Using a luciferase reporter system, AOX also had no effect on AP-1-dependent transcription in proliferating *Drosophila* (S2) or mammalian (HEK293-derived) cells. The migration of AOX-expressing iMEFs compared with that of control iMEFs was differentially stimulated by antimycin A but not by other drugs that affect mitochondrial electron flow, ATP production, reactive oxygen species (ROS), or membrane potential. We thus propose increased mitochondrial heat production as the most likely mechanism by which AOX promotes cell migration.

Conditions for AOX activation. Clearly, to affect cell motility or any other phenotype, AOX needs to be enzymatically active, modifying the cellular metabolic state. Previous studies in model systems (13–19) found that AOX expression has only minimal effects on phenotype under standard physiological conditions. Its activation requires that its substrate, ubiquinol, accumulate to substantial levels in the reduced form (44, 45), due to respiratory chain inhibition or overload. Other aspects of metazoan AOX

regulation remain largely unknown. In plants, AOX is activated by pyruvate (44, 46), although this regulation has not been demonstrated in the metazoan enzyme (47). Nevertheless, AOX rescue of cleft thorax and promotion of iMEF motility imply that it can become activated at the margin of a migrating cell sheet. Cell movement is an energy-requiring process, and AMP-activated protein kinase (AMPK), one of the cell's key regulators of energy homeostasis (48), is locally activated at the leading edge of a migrating cell sheet (49), where it promotes the movement of mitochondria into this subcellular domain. This metabolic microenvironment may also favor AOX activation, if the extra demand exceeds the capacity of respiratory complex III. In addition to AMPK, the mitochondrial unfolded protein response (mtUPR), which has previously been linked with JNK signaling (50), may be elicited. Treatment with antimycin A, which was shown here to differentially stimulate the migratory behavior of AOX-expressing iMEFs, should lead to the further activation of AOX, since blockade of cIII increases the ratio of ubiquinol to ubiquinone, diverting electron flow through AOX.

The proteins used for immortalization of MEFs, namely, the HPV16 E6 and E7 oncoproteins, may also synergize with activated AOX. Among other effects (51), E7 switches energy supply from respiration to glycolysis by activating pyruvate kinase (52). Switching to the high-capacity glycolytic pathway may underlie the increased migratory capacity of immortalized MEFs, as inferred previously for cancer cells (53, 54). AOX may enhance this by increasing pyruvate clearance via the tricarboxylic acid cycle, promoting glycolytic flux. Similarly, E6 promotes glucose transport (55–57) and facilitates the degradation of p53 (58), providing a possible link to AP-1 and JNK (59–62). E6 also upregulates hypoxia-inducible transcription factor 1α (HIF-1α) (63) and mTORC1 (64).

Is AP-1 the target of AOX? Reporter assays indicate that AP-1 and its transcriptional activity are not a direct target of AOX, although the *Drosophila* findings and the effects of inhibitor V leave open a possible direct involvement of JNK, acting through other targets.

The transcriptional reporter cell assays were conducted in proliferating cells, where AP-1 may act differently than in a migrating cell sheet. It plays diverse physiological roles, and each of its subunits is encoded by a gene family (65). Although its canonical members, c-Fos and c-Jun, drive cell proliferation (66), others are functionally diverse and are regulated by a host of mechanisms (67), including subunit composition (68), other MAP kinases (69), and mitochondrial ATP depletion (70).

The interpretation of experiments using JNK inhibitors rests upon assumptions regarding their specificity, but the effects of SP600125 and JNK inhibitor V do not fit previous assumptions regarding their mode of action. Inhibitor V was the only drug tested which blocked the migration-promoting effects of AOX in iMEFs, indicating that it acts at a step different from that influenced by AOX. These drugs have previously been studied almost exclusively in highly artificial *in vitro* systems (49, 71), taking little account of their demonstrated activity against other kinases (71–73). The alternative approach of using genetic ablation of JNK to elucidate the AOX-responsive step cannot easily be applied in mammalian cells, since JNK has diverse isoforms created by alternative splicing and is encoded by a multigene family, some of whose members have undoubtedly been functionally redeployed during evolution (74).

AP-1 is subject to oxidative inactivation (65, 75), from which it is protected in *Drosophila* by the coactivator MBF1 (76), and the loss of MBF1 sensitizes animals to cleft thorax, if also exposed to H_2O_2. AOX decreases net ROS production from mitochondria (15, 77), which could compensate for the impaired upstream activation of AP-1, but, as discussed below, its effects on mammalian cell migration were not influenced by drugs that alter ROS (MitoQ, *N*-acetyl cysteine; Fig. 8).

AOX and other signaling pathways. AOX may also act through other pathways independently of or in conjunction with AP-1. For example, immortalized fibroblasts show an enhanced induction of HIF-1α under hypoxia (78), while AOX also blunts the hypoxia response (79). In cancer cells, mitochondrial ROS activates many transcription

factors (80), and interactions between AMPK and AP-1 have been reported in cardiomyocytes (81) and Jurkat T cells (82). Mitochondrial energy status also governs calcium transport into mitochondria, which has major effects on cell migration (83).

Other transcription factors mediate mitochondrial responses to stress, notably, Nrf2 (84), ATF4 (85), and other AP-1 paralogs. Although stressors that activate AP-1 via JNK have only minimal effects on Nrf2- or ATF4-induced transcription (86), this does not exclude the possibility of the converse. JNK can be activated by mitochondrial ROS in ischemia-reperfusion (87) or by the mtUPR (50). JNK itself has also been reported to interact directly with mitochondria, triggering downstream effects (88).

AOX stimulates migratory behavior by a mechanism connected to metabolism. Of the metabolic inhibitors tested, only antimycin A preferentially stimulated the migratory behavior of AOX-expressing cells compared with control cells in the wound-healing assay. This result provides a strong clue as to the mechanism of action of AOX. The ineffective treatments included agents that should increase mitochondrial membrane potential (oligomycin [89]) or decrease it (FCCP [90], rotenone [91]), other treatments that inhibit mitochondrial ATP production (oligomycin, FCCP, and rotenone) or drugs that increase ROS production (oligomycin [92], rotenone [91, 93]) or dampen it (*N*-acetyl cysteine [94], MitoQ [95]) or that can have effects in either direction, depending on the dose (FCCP [96–98]). Agents that limit proteolytic turnover of cellular components in lysosomes (chloroquine [99]; E64d [100]) or by caspases [carbobenzoxy-valyl-alanyl-aspartyl-(*O*-methyl)-fluoromethylketone (Z-VAD-FMK) (101)] also appeared to have no differential effect on the migration of AOX-expressing cells. Not only did these other treatments fail to influence control and AOX-expressing cells differentially, but none of them produced substantial alterations to the rate of migration of control cells.

Since AOX-expressing iMEFs continue to respire when treated with antimycin A, most metabolites should be only minimally affected. The biggest difference should be in the rate of mitochondrial ATP production, when respiratory complexes III and IV are replaced by the non-proton-motive AOX. However, as indicated above, mitochondrial ATP production as such cannot be the crucial factor determining the rate of migration.

In the presence of antimycin A, the energy released by AOX in catalyzing quinol oxidation is instead converted to heat. We earlier showed that AOX-driven respiration in human cells was able to maintain the same mitochondrial temperature with less than half the amount of respiratory flux (102). In the iMEFs tested, the capacity for AOX-mediated respiration was sufficient to maintain respiratory electron flow in the presence of antimycin A at the same rate as in untreated cells (Fig. 8C). This implies that the cells should be able to maintain normal redox homeostasis in the presence of the drug with only a minimal metabolic disturbance, arising from the need to make more ATP through nonmitochondrial pathways. Maintenance of respiratory flux but in which flux is driven through AOX rather than cIII implies a pronounced thermogenic effect. Thus, we propose increased mitochondrial heat production as the most likely mechanism whereby AOX stimulates cell migration.

Fibroblast motility has long been known to be temperature dependent (103), and actin polymerization itself is highly affected by changes in temperature (104). The migration of mesenchymal stem cells is stimulated by increased expression of heat shock protein 90 (105), which impinges on various signaling pathways, possibly underlying the different effects of JNK inhibitors with poor selectivity. Heat shock protein 70 has also been shown to promote cell migration, by acting as a chaperone for the delivery of proteins needed by migrating cells to the leading edge (106). Even a transient heat shock can promote cell migration in cancer cell lines (107), which may (108) or may not (107) depend on heat shock transcription factor 1 (HSF1).

Together, these data strongly suggest that AOX activation is able to promote cell migration by raising the intracellular temperature, most likely in specific subcellular compartments. The compensatory effects on JNK signaling are thus implied to be indirect, accounting also for the fact that AOX was able to correct cleft thorax in a completely different model generated by deranged nuclear receptor signaling (8).

Further validation of this hypothesis will require the development of quantitatively reliable methods to measure the intracellular temperature in specific cell compartments *in situ*. It will require even more sophisticated technology to make such measurements *in vivo* in the *Drosophila* pupa. Alternatively, if some other metabolic effect of AOX-driven respiration is responsible, we would need to wait for metabolomic technologies to advance to the single-cell level (109) to identify plausible candidates, although novel *in situ* methods would be needed to take this to the subcellular level.

Developmental context of mitochondrial effects on cell migration. Insect metamorphosis is fuelled by stored nutrients accumulated during larval growth, principally, lipid which is metabolized in mitochondria (110). A drop in the ATP level due to nutritional limitations should activate AMPK, so as to refocus resources onto ATP production. However, because all protein kinases depend on ATP as a substrate, ATP deficiency should restrain other regulatory kinases and limit ATP-consuming developmental processes, such as cell migration. Assuming that JNK operates as one such pathway, its inappropriate downregulation in the dorsal thoracic epithelium under conditions where the ATP supply is adequate could restrict cell migration while other developmental processes are energized normally, resulting in the specific failure of midline closure.

Rapid wound healing is also important for limiting pathogen invasion. Pathogens may deplete the nutritional environment of a wound, potentially jeopardizing the processes that support tissue repair. Rapid and efficient wound closure in a nutritionally limited environment may therefore depend on metabolic remodeling to support motility (111).

A full elucidation of the processes that link mitochondrial perturbations with cell migration should be of considerable medical importance and might even enable the design of new and more effective treatments, e.g., for metastatic tumors, tissue injuries, and congenital midline closure defects. Confirmation of a role in these processes for mitochondrial heat production may open up entirely new avenues for therapy.

MATERIALS AND METHODS

***Drosophila* strains and culture.** The *Drosophila* strains used in the study and their sources are summarized in Table S1 in the supplemental material. Flies were maintained in standard high-sugar medium (15) on a 12-h light/12-h dark cycle at 25°C, except where indicated in the figure legends. Crosses were generally implemented in triplicate, with flies being tipped into new vials on three successive days after mating.

Cell culture. *Drosophila* strain S2 cells were maintained as described previously (112). AOX-positive and -negative mouse embryonic fibroblasts (MEFs) (113), sourced either from embryos transgenic for *C. intestinalis* AOX, inserted at the *Rosa26* locus (18), or from their nontransgenic littermates, were studied at passage 6. MEFs were immortalized (iMEFs) by retroviral transduction with HPV16 oncoproteins E6 and E7 (39) and maintained at 37°C in 5% CO_2 in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 20% (primary MEFs) or 10% (iMEFs) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin-streptomycin (Lonza), and 4 mM L-glutamine (Sigma-Aldrich). An AP-1 reporter HEK293 recombinant cell line (JNK signaling pathway; BPS Bioscience), here abbreviated HEK-AP1, was maintained at 37°C in 5% CO_2 in minimal essential medium (HyClone) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 1% nonessential amino acids (HyClone), 1 mM sodium pyruvate (Sigma-Aldrich), 1% penicillin-streptomycin (Lonza), and 400 μg/ml Geneticin (Gibco, Life Technologies). Geneticin was freshly distributed to each plate when cells were passaged. BJ-5ta human fibroblasts (ATCC CRL-4001) were grown at 37°C in 5% CO_2 in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 20% medium 199 (Sigma-Aldrich), 1% penicillin-streptomycin (Lonza), and 4 mM L-glutamine (Sigma-Aldrich). HEK-AP1 and BJ-5ta cells expressing *C. intestinalis* AOX or the mutated, catalytically inactive variant mutAOX (43) were obtained by pWPI-based lentiviral transduction as described previously (114). Transduced cell populations were sorted according to GFP fluorescence using a BD FACSAria II cell sorter equipped with an 85-μm nozzle and operated at a sheath pressure of 45 lb/in^2 and then cloned at limiting dilution. Clones were reverified for GFP fluorescence by fluorescence-activated cell sorting and for AOX functionality by respirometry, as described previously (43).

Wound-healing and single-cell migration assays. Samples of 90,000 cells were plated on 24-well plates (CellStar; Greiner Bio-One) as technical triplicates (3 wells per sample). After 24 h, cells were treated with one of the following reagents: 0.2% dimethyl sulfoxide (DMSO; Hybri-Max; Sigma-Aldrich), 20 μM SP600125 (Sigma-Aldrich) in 0.2% DMSO, 20 μM JNK inhibitor V (Merck), or 20 nM phorbol myristate acetate (PMA; Sigma-Aldrich) in fresh medium. After 2 h, a linear scratch was made in the center of each well with a p10 (1- to 10-μl) pipette tip. The cells were then washed three times with medium to remove detached cells, and fresh medium containing the appropriate reagent was added to each well. For testing

the effects of metabolic inhibitors in this assay, cells were seeded at 45,000 to 90,000 cells per well on 24-well plates until a monolayer was formed (24 to 48 h), before the scratch was made. After rinsing once with normal medium, medium was replaced with 1 ml of fresh medium containing one of the following drugs: oligomycin (1 ng/ml), antimycin A (60 ng/ml), rotenone (150 nM), FCCP (10 μM), *N*-acetyl cysteine (5 mM), chloroquine (20 μM), E64d (10 μg/ml), MitoQ (250 nM; a kind gift of Mike Murphy, MRC Mitochondrial Biology Unit, Cambridge, UK), valinomycin (10 μM), Z-VAD-FMK (20 μM), or just 4 μl DMSO as a solvent control. All chemicals were from Sigma-Aldrich, except where stated otherwise. To measure single-cell migration, samples of 15,000 cells were plated on 6-well plates (CellStar; Greiner Bio-One) as technical triplicates (3 wells per sample). To minimize plate-specific effects, each plate contained both AOX-positive and -negative iMEFs in all assays. Cells were imaged as described below.

Microscopy. Imaging of fly thoraxes used a Nikon digital DS-Fi1 high-definition color camera with a Nikon SMZ 745T stereoscopic zoom microscope operated by NIS-Elements D (version 4.20) software. For live imaging of *Drosophila* embryos, eggs were collected from grape juice-agar plates after adult flies were placed in a mating chamber for 1 h, covered by a dark box, dechorionated using double-sided tape (115), placed into 35-mm glass-bottom microwell dishes (MatTek Corporation) filled with Halocarbon Oil 700 (Sigma-Aldrich), and imaged for GFP fluorescence using an Andor spinning disc confocal microscope equipped with an Andor Neo 5.5 sCMOS vacuum-cooled camera at $\times$20 magnification. The larvae were cleaned of adherent food using a soft paintbrush, dried on a precision wipe, placed on an ice-cold microscope slide (Menzel Gläser), and covered with a few drops of 50% ice-cold glycerol and then with a cover glass (thickness number, 1½; Zeiss). Samples were placed in a −20°C freezer for 15 min to immobilize the larvae. Live imaging used a Zeiss Axio Imager M2 upright microscope with a Zeiss EC Plan-Neofluar 5×/0.16, WD 18.5-mm air objective and ZEN software without the ApoTome function. Pupae were gently detached from the vial, placed with the dorsal side down into 35-mm glass-bottom microwell dishes (MatTek Corporation), and imaged as described above for embryos. Wound closure time-lapse images were taken with a ChipMan Technologies Cell-IQ observation incubator equipped with a Retiga EXi 1392 charge-coupled-device camera, using a Nikon CFI Plan Fluorescence DL objective (magnification, $\times$10) until the wound closed (approximately 48 h) or for just 24 h when metabolic inhibitor drugs were applied. Images were taken every 30 min. The incubator environment was held at a temperature of 37°C and had an atmosphere of 5% CO_2, 19% O_2, and 76% N_2. Wound-healing analysis was performed using a Cell-IQ analyzer (version 4.3) by manually tracking the gap area, based on lines drawn along the wounded area of an image taken every second hour. The speed of the collective motion of the cells was measured in the most linear part of the wound area over time. In the experiment using metabolic inhibitors, the most linear time interval for closure was also analyzed and applied across all treatments and plates. To generate images for measuring single-cell migration, cells were transferred 24 h after plating to a Nikon BioStation CT instrument equipped with a Nikon DS-1QM camera and imaged at $\times$4 magnification. The environment in the incubator was held at a temperature of 37°C with a relative humidity of 85% and a 5% CO_2 atmosphere, as described above for the wound-healing assay. Images were taken every 6 min, and movies were made using CL Quant (version 3.10) software. Movement was analyzed using CellTracker image-processing software with semiautomated migration detection (116), ignoring cells that were dying or dividing.

qRT-PCR. RNA was extracted from S2 cells (117), and quantitative reverse transcription-PCR (qRT-PCR) was performed (16) as described previously, using RpL32 mRNA as an internal standard. Primers for RpL32 and AOX were those used previously (16), and those for *hep* mRNA were TTGGTTTCTTGGGGTC GATG and TGGACTCCAAGGCCAACACT, the sequences of both of which are shown 5′ to 3′.

Transfections and luciferase reporter assays in S2 cells. Firefly/renilla luciferase dual-reporter assays in S2 cells used a cotransfection protocol (42), based on plasmids TRE-fluc, mRE-fluc, pAct-RL, pAct-Gal4, and pUAST-Hep^{act}, kindly supplied by Dirk Bohmann, University of Rochester. Transfections used either TRE-fluc (a firefly luciferase reporter with the intact phorbol-ester response element) or mRE-fluc (with the mutated response element) plus pAct-RL (renilla luciferase for transfection normalization) with or without pAct-Gal4 together with pUAST-Hep^{act} for activated transcription and with the following plasmids or controls harboring AOX, as indicated in the figure legends: pMT-AOX (a kind gift of Filippo Scialó, Newcastle University), containing the *C. intestinalis* AOX-coding sequence recloned with its natural stop codon from the original vector, pCDNA5/FRT/TO (13), as an EcoRI fragment into the copper-inducible vector pMT/V5-His B (Thermo Fisher Scientific); pAC/AOX and pAC/mutAOX (43, 112), containing, respectively, the wild-type *C. intestinalis* AOX-coding sequence and the mutated, catalytically inactive variant under the control of the constitutive actin-5C promoter plus the corresponding empty vector pAc5.1/V5-His B (Invitrogen); and pUASTattB-AOX and pUASTattB-mutAOX (43), containing the same transgenes cloned into the pUASTattB vector under the control of a Gal4-dependent promoter. Expression and induction of AOX were verified for each plasmid by transfection and Western blotting as described previously (43), prior to use in luciferase reporter assays. Transfections used the FuGENE HD transfection reagent (Promega), according to the manufacturer's instructions, at a ratio of 3 μl FuGENE per μg of DNA. For each transfection, 3×10^5 cells/ml were plated on 6- or 12-well plates. To each well was added a transfection mix consisting of FuGENE, 1 μg of each plasmid to be used (per ml of culture medium), and sterile water up to a final volume of 100 μl (6-well plates) or 50 μl (12-well plates). Transfections were carried out 1 h after plating (24 h in the case of pMT-AOX or the corresponding empty vector, with addition of 500 μM $CuSO_4$ after a further 24 h), and luciferase assays were performed 72 h after transfection. In transfection mixtures to which mitochondrial inhibitors were added, 6×10^5 cells/ml were plated on 6-well plates, and drugs were added 24 h after transfection at the concentrations shown in the figure legends. For luciferase reporter experiments in which *hep* expression was knocked down by RNAi, cells were also transfected with a dsRNA against the *hep* coding sequence, prepared by a

two-round PCR-based procedure essentially as described previously (112), but using *hep*-specific primers (shown 5′ to 3′) TGGAGGCAAAGCTCCAGGC and CGCGAACGAAGCAGCCAAGG for the first round and GAATTAATACGACTCACTATAGGGGAGACATCCGCCACCCACGCACCTTC and GAATTAATACGACTCACTATA GGGGAGATCCCATTGCCCAGGTCGCCCAG for the second round, followed by transcription using a MEGAscript T7 transcription kit (Life Technologies). Knockdown at the RNA level (to ~85%) was verified in transfected cells (112) by qRT-PCR. For combined dsRNA/reporter plasmid transfections, 1×10^5 cells were plated per well in 24-well plates. After 30 min, cells were transfected with 300 ng of each relevant plasmid and 4 μg of *hep*-specific dsRNA per well in a total volume of 100 μl. A further 4 μg of the dsRNA was added 72 h later, and luciferase assays were conducted 110 h after the initial transfection. For luciferase assays, 75 μl of suspended cells from each well was transferred in triplicate to the wells of a 96-well microplate (Lab Systems) and analyzed using a Dual-Glo luciferase assay system (Promega), according to the manufacturer's protocol. Luminescence was measured using a Thermo Labsystems Luminoskan Ascent plate reader.

Luciferase reporter assays in mammalian cells. Firefly luciferase reporter assays were carried out in HEK-AP1 cells and in AOX/mutAOX-expressing clones derived from them, as follows: 30,000 cells were plated in technical duplicate (2 wells per sample) in luminometer-compatible Nunc MicroWell 96-well plates with lids (Thermo Fisher Scientific). After 24 h, the medium was replaced with medium containing either 0.2% DMSO, 20 μM SP600125 in 0.2% DMSO, 20 μM JNK inhibitor V, or no added drug. Cells were incubated for 2 h at 37°C. For PMA treatment, a second replacement medium contained 8 nM PMA plus 20 μM SP600125 in 0.2% DMSO, 20 μM JNK inhibitor V, or no other added drug, as appropriate, and the cells were incubated for a further 6 h. Luciferase assays were carried out using the Dual-Glo luciferase assay system (Promega), according to the manufacturer's protocol, and luminescence was measured using a PerkinElmer UV/visible plate reader.

Protein analysis by Western blotting. Batches of 300,000 MEFs or HEK-AP1 cells or 250,000 BJ-5ta cells were plated on 6-well plates (CellStar; Greiner Bio-One). After 24 h, the medium was replaced with medium containing either 0.2% DMSO, 20 μM SP600125 in 0.2% DMSO, 20 μM JNK inhibitor V, 8 nM PMA, or no added drug and the plate was incubated for 2 h (or 40 min, in the case of PMA). Cells were carefully rinsed in ice-cold phosphate-buffered saline (PBS) and then scraped free on ice using a CytOne cell scraper (220 mm long, 11-mm blade) in 75 μl of resuspension buffer containing 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.8, supplemented with cOmplete, Mini, EDTA-free protease inhibitor and phosphatase inhibitor cocktails (at the manufacturer's recommended amount; Roche) and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford assay. After lysis by the addition of an equal volume of SDS sample buffer (Laemmli 2× concentrate; Sigma-Aldrich), samples were heated for 5 min at 100°C and briefly centrifuged to remove particulates, and 20 μg of each extract was loaded onto 18-well precast Any kD Criterion TGX Stain-Free protein gels (Bio-Rad), which were run and blotted as described previously (43). Blots were processed as described previously (15), but with blocking in 5% bovine serum albumin (BSA) in PBS-Tween for 1 h on a shaker and using the primary antibody phospho-c-Jun (Ser 73) rabbit monoclonal no. 3270 (1:1,000; Cell Signaling Technology) or phospho-c-Jun (Ser63) II rabbit polyclonal 9261 (1:1,000; Cell Signaling Technology), with reprobing using anti-α-actinin rabbit polyclonal C-20 (1:7,000; sc-7454-R; Santa Cruz Biotechnology). Secondary antibody was peroxidase-labeled goat anti-rabbit IgG (1:10,000; PI-1000; Vector Laboratories). The chemiluminescence of all blots was documented both with film and by using a Bio-Rad ChemiDoc imager.

Respirometry. Whole-cell and permeabilized cell respiration was measured essentially as described previously (118). iMEFs were seeded 24 h before the experiment and grown in DMEM containing 4.5 g/liter glucose, 10% fetal bovine serum (Thermo Fisher Scientific), 2 mM GlutaMAX (Gibco), and 100 U/ml penicillin plus 100 μg/ml streptomycin (Lonza). To activate cell respiration, the growth medium was replaced 1 h before the assay. Cells were detached with 0.05% trypsin and counted by trypan blue exclusion. Mitochondrial respiration in permeabilized cells was assayed using an Oroboros oxygraph-2K oxygraph (Oroboros, Innsbruck, Austria), with 2×10^6 iMEFs being directly suspended in the oxygraph chamber containing 2 ml of respiration buffer B (10 mM KH_2PO_4, 20 mM HEPES-KOH, 20 mM taurine, 0.5 mM EGTA, 3 mM $MgCl_2$, 1 mg/ml essentially fatty acid-free BSA, 60 mM potassium-lactobionate, 110 mM mannitol, 0.3 mM dithiothreitol, pH 7.1). After measuring endogenous whole-cell respiration, substrates and inhibitors were added in the following order: (i) digitonin (30 μg), to permeabilize the cells; (ii) sodium pyruvate (to 5 mM), sodium glutamate (to 5 mM), and sodium malate (to 2 mM) as a cI-linked substrate mix, followed by ADP (to 2 mM); (iii) rotenone (to 150 nM) followed by succinate (to 10 mM) as a cII-linked substrate mix; (iv) antimycin A (to 30 ng/ml), to reveal AOX-mediated respiration; (v) *n*-propyl gallate (nPG; to 200 μM), to reveal any residual non-AOX-mediated oxygen consumption to be subtracted; (vi) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; to 1 mM) plus sodium L-ascorbate (to 2 mM) as a cIV-linked substrate mix; and (vii) sodium azide (to 40 mM), to reveal any non-cIV-mediated oxygen consumption to be subtracted. O_2 consumption (in picomoles · second^{-1} · milliliter^{-1}) was normalized to the amount of total proteins extracted from 1×10^6 cells and assayed by the Bradford method (119). All chemicals were purchased from Sigma-Aldrich.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00110-18.

SUPPLEMENTAL FILE 1, XLS file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLS file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLS file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLS file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLS file, 0.1 MB.

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Expression of the Alternative Oxidase Influences Jun N-Terminal Kinase Signaling and Cell Migration

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Supplemental material

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Table S1

Drosophila strains used in the study

Notes

1. VDRC = Vienna Drosophila Resource Centre
2. genotypes in Bloomington format for clarity (no italics, square brackets instead of superscripts)
3. two versions of pnr-GAL4 driver, denoted (i) and (ii) as in figure legends

Strain description	Genotype	Short name as used here	Source including catalog number if from Stock Center	Original reference if known
Multiple balancer line	w^-;If/CyO;TM3,Sb/TM6,Tb		Mika Rämet, University of Tampere	
pnr driver (ii)	(i) pnr[MD237], incorporating also UAS-GFP on chromosome 3	pnr-GAL4 or pnr[MD237]	Patricia Simpson, University of Cambridge	
pnr driver (i)	(ii) y[1] w[1118]; P{w[+mW.hs]=GawB}pnr[MD237]/TM3, P{w[+mC]=UAS-y.C}MC2,	pnr-GAL4 or pnr[MD237]	Bloomington stock centre, stock # 3039	
RNAi line against msn	VDRC RNAi construct 108948, inserted on chromosome 2	KK 101517 (*msn*)	VDRC KK library, ID# 101517	120
RNAi line against bsk	VDRC RNAi construct 10555, inserted on chromosome 2	GD 34138 (*bsk*)	VDRC GD library ID# 38138	120
RNAi line against bsk	VDRC RNAi construct 108156, inserted on chromosome 2	KK 104569 (*bsk*)	VDRC KK library, ID# 104569	120
RNAi line against hep	VDRC RNAi construct 1461, inserted on chromosome 2	GD 47507 (*hep*)	VDRC GD library ID# 47507	120
RNAi line against hep	VDRC RNAi construct 1461, inserted on chromosome 3	GD 47509 (*hep*)	VDRC GD library ID# 47509	120
RNAi line against pvr	VDRC RNAi construct 101575 inserted on chromosome 2	KK 105353 (*pvr*)	VDRC KK library ID# 105353	120
RNAi line against kay	VDRC RNAi construct 1469, inserted on chromosome 3	GD 6212 (*kay*)	VDRC GD library ID# 6212	120
RNAi line against kay	VDRC RNAi construct 9042, inserted on chromosome 3	GD 19512 (*kay*)	VDRC GD library ID# 19512	120
RNAi line against Jra	VDRC RNAi construct 100582, inserted on chromosome 2	KK 107997 (*Jra*)	VDRC KK library ID# 107997	120
RNAi line against Jra	VDRC RNAi construct 4400, inserted on chromosome X	GD 10835 (*Jra*)	VDRC GD library ID# 10835	120
AOX transgenic line	w[1118];P{UAS-AOX;w^+}F6, insertion on chromosome 2	UAS-AOX[F6]	in house	15
AOX transgenic line	w[1118];P{UAS-AOX;w^+}F24, insertion on chromosome 3	UAS-AOX[F24]	in house	15
AOX transgenic line	P{tub-AOX.K}35, insertion on chromosome X	tub-AOX[35]	in house	16
AOX transgenic line	P{tub-AOX.K}50, insertion on chromosome 2	tub-AOX[50]	in house	16
X-chromosome balancer line	B[1] sc[8] y[31d] In(1)FM7	FM7	maintained long-term in house, abailable from Bloomington Stock Centre	
GFP transgenic line	w[1118];P{UAS-GFP.nls}8, insertion on chromosome 3	GFP.nls	Bloomington stock centre, stock # 4776	
puc overexpressor	not assigned, UAS-puc2A inserted on chromosome 3	UAS-puc	Alfonso Martinez-Arias, University of Cambridge	121
pnr mutant	mwh[1] jv[1] pnr[D1]/TM2	pnr[D1]	Patricia Simpson, University of Cambridge	122

Table S2

Generic examples of genetic crosses used in the experiments

Group	Female parental genotype	Male parental genotype	Purpose
i	w- ; RNAi ; UAS-AOX[F24] / Sb	w- / Y ; 2 ; pnr-GAL4 / Tm3Ser	test rescue of homozygous RNAi constructs on chromosome 2
	w- ; RNAi ; GFP.nls / Sb	w- / Y ; 2 ; pnr-GAL4 / Tm3Ser	
	w- ; RNAi ; UAS-AOX[F24] / Tb	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	
ii	w- ; UAS-AOX[F6] / CyO ; RNAi	w- / Y ; 2 ; pnr-GAL4 / Tm3Ser	test rescue of homozygous RNAi constructs on chromosome 3
iii	w- ; RNAi / CyO ; GFP.nls / Sb	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	test rescue of heterozygous RNAi constructs on chromosome 2
	w- ; RNAi / CyO ; UAS-AOX[F24] / Sb		
iv	RNAi / FM7 ; UAS-AOX[F6] / UAS-AOX[F6] ; 3	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	test rescue of RNAi construct on chromosome X
	RNAi / FM7 ; 2 ; 3	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	
v	w- ; UAS-AOX[F6] / CyO ; UAS-puc	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	test rescue of puc OE driven by construct n chromosome 3
	w- ; GFP.nls / CyO ; UAS-puc	w- / Y ; 2 ; pnr-GAL4 / Tm3Sb	
vi	tub-AOX[35] ; tub-AOX[50] ; pnr[MD237] / TM3Sb	tub-AOX[35] / Y ; tub-AOX[50] ; pnr[D1] / TM3Sb	test rescue of compound heterozygous mutants of pnr
	w- ; UAS-AOX[F6] / CyO ; pnr[MD237] / TM3Sb	w- / Y ; pnr[D1] / TM3Sb	

Notes .

1. genotypes abbreviated as in Table S1, with superscripts shown instead in square brackets for clarity

Table S3				
Dose-response to JNK modulators				
PMA		concentration	normalized luninescence	
n=3			mean	SD
		untreated	1.00	
		2 nM	2.34	0.50
		4 nM	4.92	0.74
		6 nM	6.93	1.62
		8 nM	11.60	1.35
		10 nM	**17.78**	1.91
		50 nM	24.64	1.99
JNK inhibitor V (plus 10 nM PMA)				
n=3				
		PMA only	1.00	
		5 µM	0.85	0.092
		10 µM	0.63	0.040
		20 µM	0.16	0.050
		50 µM	0.02	0.003
Notes				
1. normalization to the conditions shown in yellow bkd				

Table S4

Repeats of experiments shown in Fig. 7

Notes
1. values used for normalization indicated with yellow bkd
2. missing data, transfections failed (all values 2 orders of magnitude lower)
3. ANOVA with Tukey HSD correction, ** p < 0.01, * p < 0.05

Repeat of Fig. 7A
Description = pMT-AOX (or empty vector) Cu induced, with intact or mutated RE reporters, pAct-RL for normalization +/- p+UAST-Hep[act] and pAct-Gal4 for JNK activation

	intact RE				mutated RE			
	AOX act	control act	AOX basal	control basal	AOX act	control act	AOX basal	control basal
mean	14.24	13.95	2.78	2..09	1.10	[note 2]	1.07	1.00
SEM	0.16	0.10	0.07	0.05	0.01		0.01	0.03

ANOVA: intact RE, act v. all other classes **; intact RE, basal v. all other classes **

Repeat of Fig. 7B
Description = pAC/AOX (or empty vector), with intact or mutated RE reporters, pAct-RL for normalization +/- p+UAST-Hep[act] and pAct-Gal4 for JNK activation

	intact RE				mutated RE			
	AOX act	control act	AOX basal	control basal	AOX act	control act	AOX basal	control basal
mean	22.20	17.50	1.90	1.70	1.30	1.60	0.90	1.00
SEM	2.40	0.70	0.10	0.10	0.01	0.01	0.01	0.01

ANOVA: intact RE, act v. all other classes **

Equivalent experiment to Fig. 7C, using pAC/AOX and pAC/mutAOX instead of the pUAST constructs
Description =pAC/AOX (or empty vector), with intact or mutated RE reporters, pAct-RL for normalization +/- p+UAST-Hep[act] and pAct-Gal4 for JNK activation

	intact RE			
	AOX act	mutAOX, act	AOX basal	mutAOX,basal
mean	15.20	18.09	1.06	1.00
SEM	3.97	0.46	0.03	0.02

ANOVA: all act v. all basal **

Repeat of Fig. 7D
Description = pAC/AOX (or empty vector), with intact or mutated RE reporters, pAct-RL for normalization +/- Hep KD by RNAi

	intact RE				mutated RE			
	AOX KD	control KD	AOX basal	control basal	AOX KD	control KD	AOX basal	control basal
mean	0.97	0.96	1.26	1.00	0.30	0.31	0.32	0.25
SEM	0.08	0.08	0.03	0.03	0.04	0.02	0.01	0.01

Table S5

Validation of HEK-AP1 AOX and mutAOX clones by respirometry

Notes

1. all clones were pre-selected by FACS, based on fluorescence of co-transduced marker GFP (see examples below)
2. resprometry performed only once for each clone, but in all cases unambiguous and as predicted
3. all values in µmol O2 per min per 10exp6 cells

type	cell-line ID	ci-linked	cII-linked	AOX-dep	cIV-linked
control cells	HEK AP1	4.26	5.54	0	16.96
AOX clones					
	AOX7	3.92	5.96	5.18	14.70
	APX12	3.30	5.78	5.50	19.66
	AOX34	4.16	5.68	4.84	18.56
	AOX37	3.16	5.76	5.02	15.42
mutAOX clones					
	mut6	3.16	5.64	0	16.66
	mut8	1.56	3.18	0	8.56
	mutGx	2.44	4.84	0	11.90
	mutXX	5.22	7.28	0	16.84

Description = S2 cells with intact RE reporter, pAct-RL and pAct-Gal4 for activation and various doses of mitochondrial inhibitors

Notes

1. values used for normaliztion indicated with yellow bkd
2. ANOVA with Tukey HSD correction, ** p < 0.01, * p < 0.05
3. showing comparisons with untreated cells; other comparisons omitted for clarity

Repeat 1		antimycin A	FCCP	H2O2	oligomycin	rotenone	no drug
		2.5 ng/ml	2 µM	44 µM	100 nM	5 nM	
	mean	4.67	1.28	1.27	5.63	1.55	1.00
	SD	0.03	0.02	0.01	0.19	0.02	0.02
	ANOVA	**	**	*	**	**	
	see note 3						
Repeat 2		antimycin A	FCCP	H2O2	oligomycin	rotenone	no drug
		0.5 ng/ml	1 µM	5 µM	50 nM	2.5 nM	
	mean	2.00	1.53	1.09	5.40	1.41	1.00
	SD	0.04	0.04	0.02	0.08	0.01	0.02
	ANOVA	**	**		**	**	
	see note 3						
Repeat 3		antimycin A	FCCP	H2O2	oligomycin	rotenone	no drug
		0.5 ng/ml	1 µM	5 µM	50 nM	2.5 nM	
	mean	1.82	1.78	1.02	4.44	1.19	1.00
	SD	0.46	0.07	0.05	0.78	0.02	0.03
	ANOVA				**		
	see note 3						